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Viral Fitness and Biological Characteristics of Highly Divergent FeLV-A Variants

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Abbreviations

104C1: Guinea pig cell line of fibroblasts
ALV: Avian leucosis virus
BLAST: Basic Local Alignment Search Tool
Bp: Base pairs
CA (p27): Capsid
CIS: Common integration site
CNS: Central nervous system
CORE: Simian virus 40 core enhancer
COSMIC: Catalogue of somatic mutations in cancer
CRFK: Crandell-Rees feline kidney cells
CT: Threshold cycle
CV: Coefficients of variation
DRD2: Dopamine receptor D2
E: Enhancer in the U3
EnFeLV: Endogenous FeLV
Env: Envelope Protein of FeLV
ExoFeLV: Exogenous FeLV
FAM102A: Family with sequence similarity 102, member A also known as EEIG1: early estrogen-induced gene 1
FCS: Fetal calf serum
FEA: Feline embryonic fibroblasts
FeLV: Feline leukemia virus
FIV: Feline immunodeficiency virus
FLV-1: FeLV-specific binding motif
Gag: Group-specific antigen
GaLV: Gibbon ape leukemia virus
GFI1B: Growth factor independent 1B transcription repressor
GRE: Glucocorticoid response element
GTF3C5: General transcription factor IIIC, polypeptide 5
HEK293: Human embryonic kidney cells
HeLa: Human HeLa cells
HIV: Human immunodeficiency virus

IN: Integrase
Kb: Kilo base pairs
KoRV: Koala retrovirus
LTR: Long terminal repeat
LVb: Leukemia virus factor b
MA (p15): Matrix
MBIP: MAP3K12 (Mitogen-activated protein kinase kinase kinase 12) binding inhibitory protein 1
MDCK: Madin-Darby canine kidney cells
MDTF: Mus dunni tail fibroblasts
MDS: Myelodysplastic syndrome
MOI: Multiplicity of infection
MuLV: Murine leukemia virus
NAIF1: Nuclear apoptosis inducing factor 1
NC (p10): Nucleocapsid
NF1: Nuclear factor 1
NKX2-1: NK2 Homeobox 1; Thyroid transcription factor 1
NKX2-2: NK2 Homeobox 2
ORF: Open reading frame
P: Promotor in the U3
PAX1: Paired Box 1
PBS: Primer binding site
Pit1: Solute carrier family 20 member 1 (phosphate transporter)
Pit2: Solute carrier family 20 member 2 (phosphate transporter)
pKW: Non-parametric Kruskal-Wallis one-way ANOVA by Ranks
pMWU: Mann–Whitney U-test
PPT: Polypurine tract
PR: Protease
PRR: Proline-rich region
PUS10: Pseudouridylate synthase 10; Coiled-coil domain-containing protein 139 (CCDC139)
R: Repeat region between U3 and U5
RBD: Receptor-binding domain
RT: Reverse transcriptase

RTCGD: Retrovirus and Transposon tagged Cancer Gene Database

SA: Splice acceptor

SD: Splice donor

SNX25: Sorting Nexin 25

SPF: Specified pathogen free

ST-IOWA: Pig cell line

SU (gp70): Surface unit domain of FeLV (glycoprotein of 70 kilo-Dalton)

SUSD4: Sushi domain containing 4

TAG: Tumor Associated Gene

TCID50: Tissue culture infectious dose 50

TLR5: Toll-like receptor 5

TM (p15E): Transmembrane domain

TMPRSS5: Transmembrane protease, serine 5; SPINESIN

TRAIL: TNF-related apoptosis-inducing ligand

U3: unique 3' region of the LTR

U5: unique 5' region of the LTR

VRA: Variable region A

VRB: Variable region B

VRC: Variable region C

Ψ: Packaging signal

1 Abstract

In a cat (#261) that had ostensibly recovered from experimental feline leukemia virus (FeLV) infection, we observed virus reappearance associated with development of B-cell lymphoma. The virus variants found in this cat had largely replaced the original virus strain (FeLV-A/Glasgow-1) and had heavily mutated *env* genes and long terminal repeats (LTR). The goals of this study were to characterize the virus variants for viral fitness and host range and determine proviral integration sites. FeLV-A/Glasgow-1 was sequenced and chimeric viruses containing *env* or LTR of the progeny viruses were constructed. The recombinant viruses showed lower or similar replication efficiency in feline embryonic fibroblasts (FEA cells) compared to FeLV-A/Glasgow-1 when FeLV p27 antigen but less so when viral RNA was quantified. The host range of the FeLV-A/261*env* variant was ecotropic confirming the FeLV-A subgroup. Using genome walking, integration sites of the progeny proviruses in cat #261 and a second FeLV-A/Glasgow-1 infected cat with a T-cell lymphoma analyzed for comparison were found near genes that may contribute to neoplastic transformation (i.e. *GFI1B*, *NAIF1, NKX2-2, PAX1*, *NKX2-1*). All integration sites were distinct from common integration sites described so far in FeLV-infected cats as well as distinct between the two cats. This is the first study to document integration sites of FeLV-A/Glasgow-1 and progeny viruses thereof.

Keywords: FeLV, FeLV-A variants, host range, replication efficiency, integration sites

2 Zusammenfassung

Eine mit dem felinen Leukämievirus (FeLV) infizierte Katze (#261) mit regressivem Infektionsverlauf, zeigte eine Reaktivierung der Infektion und die Entwicklung eines B-Zell Lymphoms. Die auftretenden Virusvarianten hatten das ursprüngliche Virus (FeLV-A/Glasgow-1) beinahe vollständig ersetzt und zeigten ungewöhnlich viele Mutationen im Hüllprotein (*env*)-Gen und den Long Terminal Repeats (LTRs). Das Ziel dieser Studie war die Replikationsfähigkeit, das Wirtsspektrum und die Integrationsstellen der Virusvarianten zu charakterisieren. Dazu wurde FeLV-A/Glasgow-1 sequenziert und Virus-Chimären mit *env* und LTRs der Varianten hergestellt. Die rekombinanten Viren zeigten eine im Vergleich zu FeLV-A/Glasgow-1 schwächere oder vergleichbare Replikationsfähigkeit in felinen embryonalen Fibroblasten (FEA Zellen), v.a. wenn p27-Antigen und etwas weniger, wenn virale RNA gemessen wurde. Das Wirtsspektrum der rekombinanten FeLV-A/261*env* war ecotrop, was die Zugehörigkeit dieser Variante zur FeLV-A Untergruppe bestätigte. Mithilfe der „Genome Walking“ Methode wurden die Integrationsstellen bei der Katze #261 und einer zweiten FeLV-A/Glasgow-1 infizierten Katze mit einem T-Zell Lymphom in der Nähe von potentiell mit Tumorgenese assoziierten Genen lokalisiert (*GFI1B*, *NAIF1, NKX2-2, PAX1*, *NKX2-1*). Alle Integrationsstellen sind erstmals beschrieben und unterscheiden sich zwischen beiden Katzen. Die Studie demonstriert erstmals Integrationsstellen von FeLV-A/Glasgow-1 und Varianten dieses Virus.

Schlüsselwörter: FeLV, FeLV-A Varianten, Wirtsspektrum, Replikationsfähigkeit, Integrationsstellen

3 Introduction

Feline leukemia virus (FeLV) is a virus found in domestic cats and other felids, such as the Iberian lynx, Florida panther and captive born jaguarundis (Meli et al., 2009, Brown et al., 2008, Cunningham et al., 2008, Filoni et al., 2012, Luaces et al., 2008). FeLV is part of the retrovirus family and grouped in the genus gammaretrovirus together with retroviruses of rodents (e.g. MuLV), gibbons (GaLV) and koalas (KoRV). Jarrett et al. first described the virus in a study about transmissible leukemia (Jarrett et al., 1964b, Jarrett et al., 1964a).

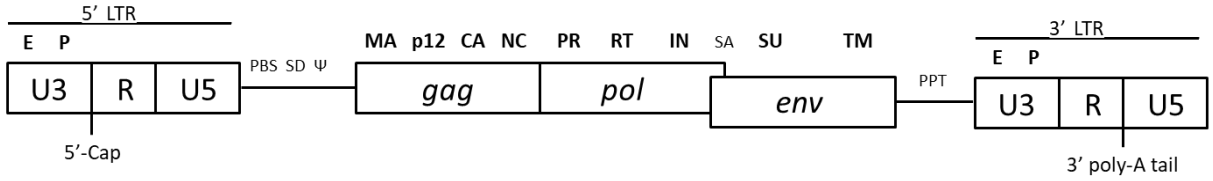

Figure 1: Genomic organization of FeLV provirus. Adapted from (Maetzig et al., 2011). For explanation of the indicated abbreviations see text.

Figure 1 shows the proviral genomic structure of FeLV containing three open reading frames (ORFs: *gag, pol* and *env*), which are flanked by the 5' and 3' long terminal repeats (LTR). The *gag* (group-specific antigen) gene is translated into a polyprotein Pr65gag, which is the precursor of the structural proteins; the matrix (MA or p15), the p12 (probably involved in the integration process), the capsid (CA or p27) and the nucleocapsid (NC or p10). The *pol* (polymerase) encodes the viral enzymes, being the protease (PR), the reverse transcriptase (RT) and the integrase (IN). The *env* (envelope) gene encodes the glycoproteins surface unit (SU or gp70) and the transmembrane (TM or p15E) domains. The LTRs are part of the non-coding region of the genome and are separated into the U3 (unique 3' region), R (repeat region) and U5 (unique 5' region). U3 contains the enhancer (E) and the promotor (P). In the viral genomic RNA both LTRs are incomplete, without the U3 but with a capping at the 5' side and without the U5 but a poly-A tail at the 3' side. LTRs are completed during the reverse transcription process. Other non-coding RNA regions are the primer binding site (PBS), the splice donor (SD), the packaging signal (Ψ), the splice acceptor (SA) and the polypurine tract (PPT).

The retroviral replication cycle begins with the attachment of the envelope glycoproteins to a host cell receptor followed by a membrane fusion releasing the virion core into the host cell. In the cytoplasm the enzyme reverse transcriptase starts to transcribe the dimeric RNA strand into double stranded DNA (dsDNA), which is transported into the cell nucleus. With the help of a second enzyme, the integrase, this dsDNA is integrated into the host cell genome as a provirus. From the integrated provirus viral RNA is expressed either in spliced or unspliced form. The splicing of the viral RNA results in subgenomic RNAs from which *env* proteins are translated. The unspliced viral RNA serves as a template for translation of *gag* and *pol* and also for whole genome replication. Fusion proteins of *gag* and *pol* (termed Pr65gag) are transported to the plasma membrane, polymerize together and are thought to interact with the membrane phospholipids via an N-terminal myristylation (Rein et al., 1986). Co-localized *gag* proteins (Felsenstein and Goff, 1988), transmembrane *env* proteins interact with the polymerized Pr65gag proteins. The packaging process of the dimeric virus RNA is started by its packaging signal (Ψ) and interaction with the Pr65gag complex. An immature virus particle is formed, which buds from the plasma membrane acquiring the membrane bilayer interspersed with *env* glycoproteins. Maturation into an infectious virus is induced by a viral protease cleaving the Pr65gag into its components, which then form the matrix (MA), capsid (CA) and nucleocapsid (NC) (Yoshinaka et al., 1985).

There are different outcomes of infection and not every cat exposed to FeLV develops clinical signs. The majority of FeLV-exposed cats react with an effective immune response, leading to a regressive or even abortive infection with transient or undetectable viremia (Flynn et al., 2002). The regressive infection is often followed by a latent nonproductive nonviremic infection, where the virus persists in the genomic DNA of the host cells embedded as a provirus. If those provirus-positive cats are unable to maintain protective immunity, for example due to severe immunosuppression, the reemergence of the virus may occur; this is often paralleled by development of malignancies. This pathomechanism was shown in studies addressing FeLV reactivation after immunosuppression (Rojko et al., 1982, Pedersen et al., 1984, Hofmann-Lehmann et al., 2007). In some cats humoral and cellular immunity against FeLV is insufficient from the start of infection and a persistent viremia develops. Those cats with so-called progressive infection will succumb to

FeLV-associated diseases over a time course of several weeks to a few years (Hoover and Mullins, 1991, Hartmann, 2011).

There are several FeLV subgroups defined based on their differences in host cell receptor usage and interference capabilities (Sarma and Log, 1973), which have a direct impact on clinical characteristics.

FeLV-A is the main infectious agent and is transmitted through saliva, feces and blood among susceptible felids (Gomes-Keller et al., 2009, Francis et al., 1977). In experimental studies FeLV-A is strongly associated with thymic lymphoma of T-cell origin developed after a progressive infection for up to four years during which no clinical signs were observed (Rohn et al., 1994, Phipps et al., 2000). FeLV-A utilizes the thiamine transporter THTR1 as a receptor (Mendoza et al., 2006, Shalev et al., 2009). All other FeLV subgroups arise from either recombination of FeLV-A with endogenous gamma retrovirus sequences (enFeLV, ERV-DC) or mutations in the FeLV-A *env* gene. FeLV-B (Stewart et al., 1986, Overbaugh et al., 1988b) and the more recently discovered FeLV-D (Ito et al., 2015) arise from recombination with endogenous retrovirus enFeLV or ERV-DC, respectively. In case of FeLV-B, this recombination leads to acquisition of enFeLV sequences encoding proteins of the surface unit (SU), which alter the receptor usage from THTR1 to Pit1 or Pit2 (both genes are part of the Solute Carrier Family 20 encoding phosphate symporter proteins) (Takeuchi et al., 1992, Boomer et al., 1997). Changes of receptor usage have an influence on cell tropism and host range. While FeLV-A has an ecotropic host range and therefore is only able to infect feline cell lines, the altered receptor usage of FeLV-B expands its host range and the virus, thus, is able to infect a variety of mammalian cell lines including human cell lines (Shojima et al., 2006, Nakata et al., 2003). Receptors and host range for FeLV-D have not yet been identified (Ito et al., 2015).

FeLV-C and FeLV-T are formed by mutations (Neil et al., 1991, Donahue et al., 1991). FeLV-C utilizes the heme exporter FLVCR1 as a receptor (Quigley et al., 2000, Tailor et al., 1999). Since FeLV-C is only found in anemic cats it is thought that the binding of the *env* protein to the FLVCR1 receptor disrupts its function preventing early erythropoiesis and consequently leads to a red blood cell aplasia (Onions et al., 1982, Dornsife et al., 1989, Rey et al., 2008a, Quigley et al., 2004). FeLV-T is a fusion defective variant of FeLV that depends on a soluble co-factor, termed FELIX, for cell entry. This co-factor is primarily expressed by T-cells and derives from

enFeLV. It resembles the SU protein from subgroup FeLV-B, which also utilizes the Pit1 receptor (Anderson et al., 2000). FeLV-T causes severe immunosuppression in cats (Overbaugh et al., 1988a, Anderson et al., 2000, Sakaguchi et al., 2015).
The occurrence of neoplasia is very common in FeLV-infected cats and has to do with the ability of the virus to integrate itself into the host's genomic DNA. The mechanism is also described as retroviral insertional mutagenesis. If the retrovirus integrates near a proto-oncogene or an oncogene itself the promotor and enhancer function of the LTR can upregulate the gene expression and lead to tumorigenesis (Johnson et al., 2005). Another possible way of inducing tumors is integration into and thereby altering or disrupting a gene that is responsible for tumor prevention. In mice, this mechanism was instrumental in finding over a hundred loci of proto-oncogenes in murine retrovirus induced tumors and in finding genes that were closely related to these retroviral insertions. These sites were termed as common integration sites (CIS). The detection of the c-myc gene as a CIS in murine (MuLV) and avian leucosis (ALV) led also to the investigation and discovery of this gene as a CIS in FeLV induced lymphomas (Neil et al., 1984, Forrest et al., 1987, Miura et al., 1987, Miura et al., 1989). Other FeLV-integration loci include flvi-1, flvi-2 (bmi-1), fit-1, flit-1 and pim-1 (Tsatsanis et al., 1994, Levy et al., 1993, Levy and Lobelle-Rich, 1992, Levesque et al., 1990, Tsujimoto et al., 1993, Fujino et al., 2009).
In earlier studies, it was reported that up to 80% of the diagnosed lymphomas and leukemias were associated with FeLV (Francis et al., 1977, Francis et al., 1979, Hardy et al., 1980). Those percentages are decreasing though (Reinacher and Theilen, 1987, Moore et al., 1996, Teske et al., 2002, Stutzer et al., 2011) and one main reason is probably the decreasing prevalence of FeLV infection, which in turn is assigned to efficient FeLV diagnosis-removal programs and the development of efficacious FeLV vaccines (Cotter et al., 1975, Louwerens et al., 2005, Lutz et al., 2009, Meichner et al., 2012). In 1984 the first commercially available FeLV vaccine was brought to the market in the USA. The step to develop an effective vaccine was of great interest not only for the cat population but also in sight of the possibility of new findings for HIV research.
This thesis is a follow up study on the paper "Dominance of highly divergent feline leukemia virus a progeny variants in a cat with recurrent viremia and fatal lymphoma" by A Katrin Helfer-Hungerbuehler and co-workers (Helfer-Hungerbuehler et al., 2010). This study reported the case of cat #261, a female SPF cat, which was

infected with FIV and vaccinated against FeLV prior to an experimental FeLV-A/Glasgow-1 infection. The FeLV-viremia in this cat was only transient; thereafter, during later infection no antigen could be detected anymore by p27 antigen ELISA although the cat was provirus positive as assessed by a nested FeLV PCR assay (Hofmann-Lehmann et al., 2001). The reappearance of FeLV viremia was observed 8.5 years after the initial infection with FeLV-A/Glasgow-1 and was associated with immunosuppression (CD4+ T cell depletion) due to the pre- and co-existing infection with FIV. The cat developed a multicentric FeLV-positive lymphoma and was euthanized. By characterizing the progeny viruses it was discovered that the virus variants found in this cat had largely replaced the inoculation strain and had unusually heavily mutated envelopes and LTRs. In sight of the commonly highly conserved nature of FeLV-A strains these highly mutated virus variants were of special interest in terms of viral properties and replication behavior.

The goal of this thesis was thus to characterize the virus progeny variants that arose in cat #261 by assessing the viral fitness and the host range of these viruses *in vitro* in comparison with the original inoculation prototype strain FeLV-A/Glasgow-1 and to determine the viral insertion sites of FeLV-A/Glasgow-1 and its progeny variants.

4 Materials and Methods

4.1 Animal samples

Tissues provided for this study derived from two specified pathogen-free (SPF) cats. Both cats were kept under barrier conditions and group housed according to the Swiss law and optimal ethological and hygienic conditions as previously described (Geret et al., 2011). All experiments were officially approved by the veterinary office of the canton of Zurich (197/89, 43/90, 66/91, 131/91, 329/91, 56/95, 59/2005).

Cat #261 was a female SPF cat (Ciba Geigy, Basel, Switzerland) infected with a Swiss isolate of feline immunodeficiency virus (FIV) at the age of 17 weeks (Lehmann et al., 1991) and vaccinated with a recombinant FeLV p45 protein vaccine (Leucogen, Virbac, Nice, France) at the age of 41 weeks, revaccinated twice at the age of four years. In between, with 59 weeks, the cat was exposed intraperitoneally to FeLV-A/Glasgow-1 and developed a transient FeLV viremia but was then FeLV antigen and viral RNA negative until FeLV reappeared at the cat's age of 9.6 years (Lehmann et al., 1991). The cat was co-housed with FeLV p27-positive cats during the first seven years post infection, after which it was kept with only p27-negative cats. At the time of necropsy, a multicentric FeLV-positive lymphoma categorized as a monoclonal proliferation of B cells was found (Helfer-Hungerbuehler et al., 2010). Histopathological examination was performed and samples from 27 tissues were collected, snap-frozen in liquid nitrogen and stored at -80°C.

For the purpose of this study, tissues of the sternal lymph node, kidney, spleen and duodenum were used, all of which had previously been identified as part of the multicentric lymphoma.

The **cat #67** was part of a FeLV vaccination study (Hofmann-Lehmann et al., 2006) and turned FeLV-positive after exposure to FeLV-A/Glasgow-1. The cat remained persistently FeLV-positive (progressive infection). After developing a malignant T-cell lymphoma in thymus, spleen, liver and bone marrow, the cat was euthanized at the age of three years and underwent histopathological examination at which tissue samples were collected, snap-frozen in liquid nitrogen and stored at -80°C. For the purpose of this study, tumor tissue from the mediastinal lymph nodes and tissues of kidney, spleen and duodenum were used.

4.2 Nucleic acid extraction and cDNA production

Genomic **DNA** was extracted using the QIAgen DNeasy® Blood & Tissue Kit (Qiagen, Hombrechtikon, Switzerland) from tissues of the cats #261 and #67. Viral **RNA** from cell culture supernatant was extracted using the MagNA Pure LC RNA Isolation Kit - High Performance (Roche Diagnostics GmbH, Mannheim). Negative extraction controls consisting of phosphate buffered saline (PBS 10x, without Ca^{2+} and Mg^{2+}, Life Technologies™) were included with each extraction batch (Helfer-Hungerbuehler et al., 2010). Reverse transcription of RNA into cDNA was performed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Rotkreuz, Switzerland).

4.3 Serological assays

The level of FeLV antigenemia was determined by p27 antigen sandwich ELISA as described (Lutz et al., 1983). Values above 4% of the positive control run with each assay were considered positive (Hofmann-Lehmann et al., 2006).

4.4 FeLV integration site detection from tumor DNA

Genomic DNA from the sternal lymph node (very high provirus load of the *env* variants) of cat #261 and tumor tissue of cat #67 were prepared for screening of FeLV integration sites using the Universal GenomeWalker2.0 kit (Clontech Laboratories, Mountain View, CA) as described in the manufacturer's user manual. Briefly, tumor DNA was digested with restriction enzymes *Dra*I, *Eco*RV, *Pvu*II or *Stu*I to create four different DNA libraries. GenomeWalker adaptors were ligated to the different libraries DNA. A nested PCR was performed by using primers provided by Clontech, which bind to the GenomeWalker adaptor and specific primers, which bind to our area of interest, the FeLV LTR. Due to the widespread presence of endogenous FeLV in the cat genome, the method for the detection of CIS was adapted for the specific detection of only exogenous FeLV. For the *Pvu*II and *Eco*RV libraries the primers used for the primary PCR were AP1 (Clontech) and GWLTR3'-GSP1 (5'-GAG GCC AAG AAC AGT TAA ACC CCG GAT ATA G-3') and for the nested PCR AP2 (Clontech) and GWLTR3'-GSP2 (5'-CTA ACC AAT CCC CAC GCC TCT CGC TTC TG-3'). The *Dra*I and *Stu*I libraries were amplified using AP1 and AP2 PCR primers (Clontech) and GWLT5'-GSP3 (5'-AAA TGA GGC GGA AGG TCG

AAC TCT GG-3') for the primary PCR and GWLT5'-GSP4 (5'-GGG TTT AAC TGT TCT TGG CCT CAA GC-3') for the nested PCR. For primary PCRs the conditions were as follows: 1 cycle of 94°C for 15 s, 7 cycles of 94°C for 25 s followed by 72°C for 3 min, 32 cycles of 94°C for 25 s followed by 67°C for 3 min and 67°C for an additional 7 min after the last cycle. Conditions for secondary PCR: 1 cycle of 94°C for 15 s, 5 cycles of 94°C for 25 s followed by 72°C for 3 min, 20 cycles of 94°C for 25 s followed by 67°C for 3 min and 67°C for an additional 7 min after the last cycle. Purified PCR products (NucleoSpin® Gel and PCR Clean-up, Macherey-Nagel, Clontech, Saint-Germain-en-Laye, France) were either directly sent for sequencing (Microsynth, Balgach, Switzerland) or prepared for cloning. Three to five PCR products of every restriction site library were taken and ligated into pCR®4-TOPO® Vector (TOPO TA® Cloning kit, Invitrogen, Basel Switzerland) and plated using one petri dish per PCR product. One white colony from every plate was selected and incubated in 6-8 ml LB-medium (prepared by laboratory technicians) overnight followed by plasmid extraction (QIAprep Spin Miniprep Kit). The plasmids were sent in for sequencing using the primers M13 and M13r. The returned sequences were edited, assembled and analyzed with Geneious software (version 8.1, Biomatters Limited). All sequences were compared to the nucleotide collection and the Reference Sequence (RefSeq) collection of *Felis catus* genome at NCBI and at Ensemble using Basic Local Alignment Search Tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi). The detection of exogenous LTR sequences adjacent to feline genome sequences verified proviral integration events. To identify possible CIS the genes near insertion sites were compared to insertion sites of the mouse on the Retrovirus and Transposon tagged Cancer Gene Database (RTCGD) (National Cancer Institute at Frederick, 2004, Akagi et al., 2004) and to two human databases one was Tumor Associated Gene (TAG) (NCKU Bioinformatics Center, 2013, Chen et al., 2013) and the other was the catalogue of somatic mutations in cancer (COSMIC) (Wellcome Sanger Institute, 2004, Forbes et al., 2017).

4.5 Sequencing of the plasmid pFGA-5 (FeLV-A/Glasgow-1)

Since FeLV-A/Glasgow-1 had only been previously partially sequenced the plasmid pFGA-5 (Stewart et al., 1986) (kindly provided by Professor B. Willett of the University of Glasgow, GB) containing the whole genome of FeLV-A/Glasgow-1

(12,709 bp) was entirely sequenced (GenBank: KP728112.1), thus allowing better sequence comparison of FeLV-A/Glasgow-1 with its progenies and recombinant constructs. For this purpose, the primers described in Table 1 were used. All primers were commercially synthesized (Microsynth, Balgach, Switzerland).

Table 1: PCR primers used for amplification and sequencing of FeLV-A/Glasgow-1

Name	**Sequence 5'-3'**	**Length (bp)**	**Reference**
Glasgow-1/ 29F	CAGCTACTGCAGTGGTGTCATTTC	24	This study
Glasgow-1/ 29R	GAAATGACACCACTGCAGTAGCTG	24	This study
Glasgow-1/ 476R	GAGCCCCCAAATGAAAGAC	19	This study
Glasgow-1/ 770R	CCCTCGTCTCCGATCAACAC	20	This study
Glasgow-1/ 898F	GAAACCGTCATGGGCCAAAC	20	This study
Glasgow-1/ 1346R	GAGGAAGGATCAGGCGGTAAC	21	This study
Glasgow-1/ 2400F	CTCCACTCTCCTCAACTTAG	20	This study
Glasgow-1/ 2560R	GGTCACTGAGAGGTCCATCTG	21	This study
Glasgow-1/ 3249F	TTTCTGCCTGCGACTACAC	19	This study
Glasgow-1/ 4066F	CTAGGACAGCCGCTAACTATC	21	This study
Glasgow-1/ 4785F	AACCGTCTTACCCACTGAAC	20	This study
Glasgow-1/ 5059R	GGAGGTGTAAGTCTCTGTTAGG	22	This study
Glasgow-1/ 5436R	GGGTCGGTATGCACAATG	18	This study
Glasgow-1/ 5617F	CCTATGGCTCACTTCTTTGATACTGATATCTCTA	34	(Meli et al., 2009)
Glasgow-1/ 5847F	ACATATCGTCCTCCTGACGAC	21	(Chandhasin et al., 2005a)
Glasgow-1/ 6058R	CCCACCAGAAACGCTAAG	18	This study
Glasgow-1/ 6382F	TGGGGCCAAAGGGAACACAT	20	(Meli et al., 2009)
Glasgow-1/ 7266F	TACCATCAACCCGAATATGTGTACACA	27	(Meli et al., 2009)
Glasgow-1/ 7691F	CGGCAACAACTGTTTGACTCC	21	This study
Glasgow-1/ 7691R	GGAGTCAAACAGTTGTTGCCG	21	This study
pFGA1_catF	GATCATCTCAGCCAGCATGATAAAC	25	This study
Glasgow-1/ 10123R	TGATAGACGCAGCCAAGCAA	20	This study

4.6 Construction of recombinant FeLV 261 proviruses

Recombinant FeLV-A/261 proviruses were constructed by substituting the LTR or the *env* gene of FeLV-A/261 from the horizontally transmissible prototype isolate, FeLV-A/Glasgow-1 (see Figure 2) according to the method described by Chandhasin et al (Chandhasin et al., 2005a, Athas et al., 1995b). PCR was performed to amplify different LTR-sequences out of DNA from the sternal lymph node tissue of the cat #261 with the primers FeLV-LTR-F1 (5'-CAATACGATCCGGACCGACC-3') and FeLV-LTR-R1-***Sal*I** (5'-GTATCCAACG**GTCGAC**TGAAAGACCCCCGAACTAGGTC-3') including an additional *Sal*I site. The PCR product was then separated on a 1% agarose-gel. Bands of the expected size (~572 bp) were cut out and DNA was purified by QIAquick Gel Extraction Kit (Qiagen). After pCR®4-TOPO® TA cloning, colonies were sent for sequencing (Microsynth) with the primers M13 and M13r. Four different LTR-variants were obtained; two among them had been described earlier as pKH11.1 [GenBank: FJ613291] and pKH12.1 [GenBank: FJ613295] (Helfer-Hungerbuehler et al., 2010). The new variants were termed Cat261-LTR1 and Cat261-LTR3 [GenBank: KU962189 and KU962190], respectively. The clones containing the different LTR-variants were digested with *Cpo*I (*Rsr*II) and *Sal*I resulting in a fragment of approximately 572 bp. The plasmid containing FeLV-A/Glasgow-1 (pFGA-5) (see Figure 3) was digested with *Cpo*I and *Sal*I as well resulting in a backbone fragment of 11,943 bp lacking the LTR. A ligation step was performed using T4 DNA Ligase (Thermo Scientific, Reinach, Switzerland) overnight at 16°C ligating the FeLV-A/Glasgow-1 backbone with one of the four different LTR variants (FeLV-A/261L11; FeLV-A/261L12; FeLV-A/261L1; FeLV-A/261L3). Correctness of sequence ligations was verified at the beginning of the experiments by sequencing (Microsynth). All FeLV-A/Glasgow-1 LTR progenies were also re-sequenced (Microsynth) at the end of the replication study. No mutational changes occurred during the time the progenies were in cell culture.

The variant *env* SP261-III (EU359305; obtained from a spleen sample of the cat #261) was cut out from an already existing plasmid, termed p25 (kindly provided by Valentino Cattori, Clinical Laboratory; vector: pCR®II-TOPO®, Invitrogen, Paisley UK, insert: S261-III), with the restriction enzymes *Cpo*I and *Xho*I, resulting in a 2,080 bp fragment. Digestion of FeLV-A/Glasgow-1 with *Cpo*I and *Xho*I resulted in a 10,589 bp long backbone. To obtain a virus composed of FeLV-A/Glasgow-1 backbone and

SP261-III *env* the same ligation step as described above was applied. The obtained ligation product was termed FeLV-A/261*env*.

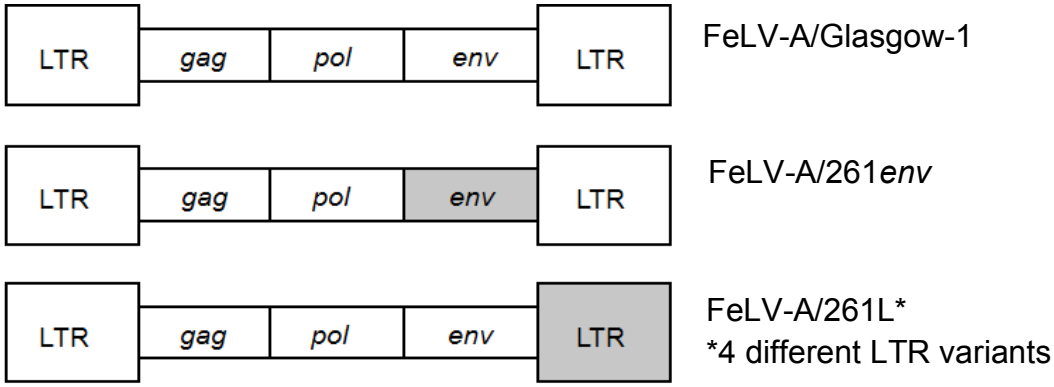

Figure 2: Diagram of recombinant FeLV proviruses constructed by replacing the envelope gene or LTR of FeLV-A/Glasgow-1 with envelope or LTRs of the variants detected during FeLV infection of cat #261 (gray shaded area). * The four different LTR variants were L1, L3, L11, L12. Adapted from (Chandhasin et al., 2005a)

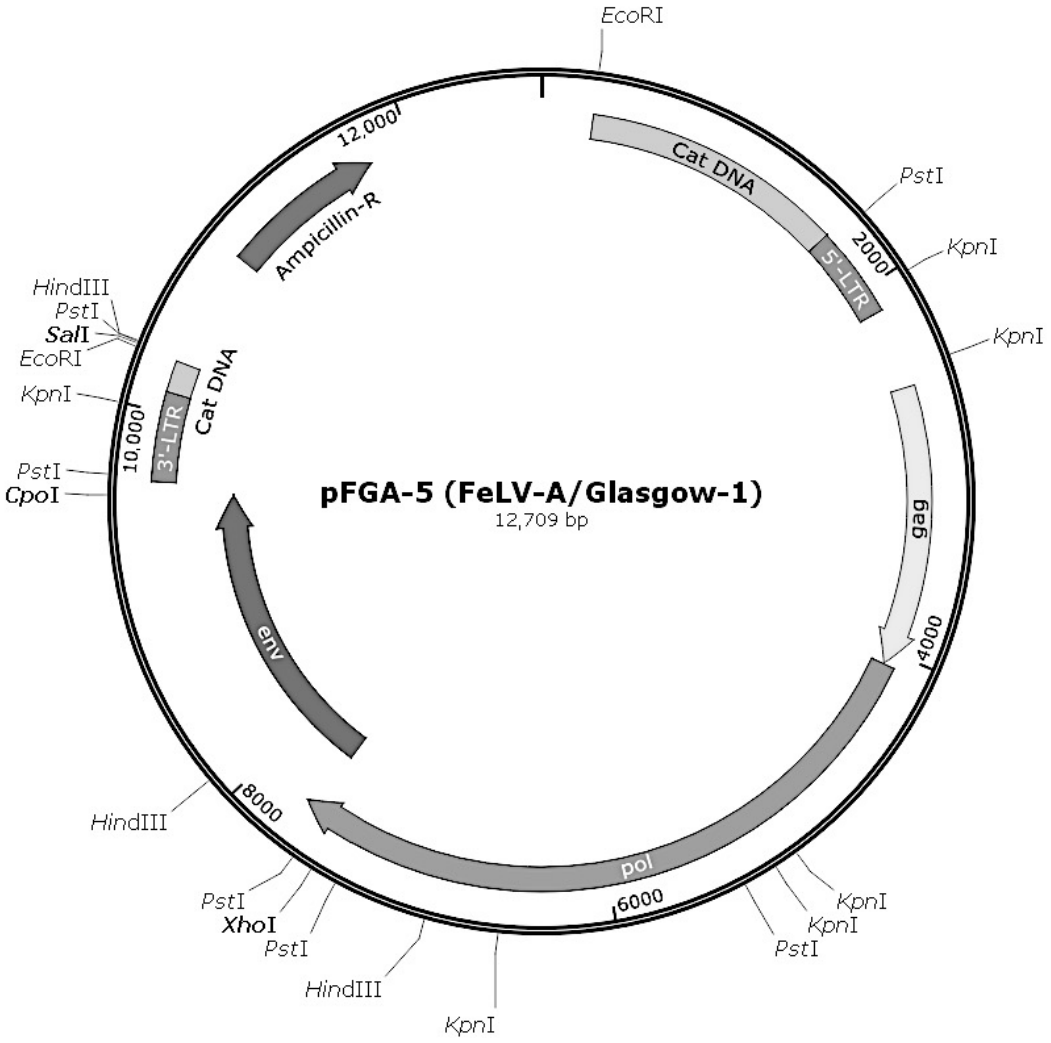

Figure 3: Map of the plasmid pFGA-5 (FeLV-A/Glasgow-1) with selected restriction sites created with SnapGene®. The restriction enzymes used in this study are in bold (***Sal***I, ***Cpo***I and ***Xoh***I).

4.7 Cell culture

All cells lines used in this study were tested for absence of FeLV infection by p27 FeLV ELISA from the cell culture supernatant (Lutz et al., 1983) and using ExoFeLV U3 real-time PCR and DNA extracted from cell pellets of the cultures (see 4.8.2).

4.7.1 Preparation of virus stocks

In order to prepare infectious viral stocks, feline embryonic fibroblasts (FEA) (Jarrett et al., 1973) were transfected with plasmid DNA containing the recombinant or the prototype FeLV-A/Glasgow-1 viruses. Transfection was performed using an optimized Lipofectamine 2000 protocol (Life Technologies™; Carlsbad, California) when cells reached a confluency of approximately 70%. Lipofectamine in Opti-Mem-Medium (Gibco by Life Technologies, Stockholm, Sweden 11058-021) was mixed with diluted plasmid DNA, added to cells seeded on a 24-well plate and incubated for 4 hours. After a washing step with Roswell Park Memorial Institute medium (RPMI; Gibco, Life Technologies,) the medium was changed to RPMI containing 10% fetal calf serum (FCS). Transfection conditions were set at a final Lipofectamine concentration of 1.5 µL per ml and a DNA concentration of 500 ng per ml. By p27 FeLV antigen ELISA from culture supernatant collected at day 5 after transfection, a productive infection was confirmed and the cells were maintained for four weeks to collect supernatant. Supernatant was aliquoted and stored at -80°C. The Tissue Culture Infection Dose 50 ($TCID_{50}$) was determined by titration as described by the Spearman & Kärber algorithm (Hierholzer and Killington, 1996). The titration was performed on 12-well plates containing 80% confluent FEA cells in 1 ml RPMI. One aliquot of stock solution of viral supernatant was diluted on ice in a tenfold serial dilution (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}). An aliquot of the virus stock and each dilution was stored at -80°C. The FEA cells were inoculated with 1 ml of every dilution in triplicates and incubated at 37°C, 5% CO_2 for two hours. After the incubation the inocula were removed and 1.5 ml RPMI complete was added to each well. Plates were maintained for one week at 37°C, 5% CO_2. At day 4 and day 7 0.5 ml supernatant from each well were collected and stored a -80°C. A p27 antigen ELISA was performed with all stored aliquots and the $TCID_{50}$ was then calculated.

4.7.2 Virus replication kinetics

FEA cells were seeded at a density of 20,000 cells per well on a 24-well plate and were transfected with the recombinant proviruses and the original challenge virus FeLV-A/Glasgow-1 as described above in quadruplicates. Supernatant was collected at days 3, 5, 7, 10, 12, 14, 17, 21 and 24. Cells were passaged every seventh day. A FeLV p27 antigen ELISA was directly performed from the supernatant collected. Quantitative PCR was performed using cDNA from viral RNA extracted from the supernatants (for details see 4.2, 4.3 and 4.8.3). At the end of the experiment the infected FEA cells were harvested, the genomic DNA extracted and the sequences of the recombinants were obtained (Microsynth) to ensure conformity with the inoculated strains.

4.7.3 Host range comparing FeLV-A/261*env* to FeLV-A/Glasgow-1

The following adherent cell lines were used in this study: feline embryonic fibroblasts (FEA) (Jarrett et al., 1973), Crandell-Rees feline kidney cells (CRFK) (ATCC CCL-94), Madin-Darby canine kidney cells (MDCK) (ATCC CCL-34), Mus dunni tail fibroblasts (MDTF) (ATCC CRL-2017), Guinea pig fibroblasts 104C1 (ATCC CRL-1405) and porcine ST-IOWA (ATCC CRL-1746). Cells were maintained in RPMI 1640 medium supplemented with 10% FCS, 1% Gibco® Antibiotic-Antimycotic containing 10,000 units/ml of penicillin, 10,000 µg/ml of streptomycin, 25 µg/ml of Fungizone® Antimycotic and 2 mM L-glutamine (Gibco, Life Technologies, 25030-081). Human embryonic kidney cells (HEK293) (ATCC CRL-1573) and human HeLa cells (ATCC CCL-2) were as well maintained in RPMI 1640 medium supplemented with 5% FCS, antibiotic-antimycotic and L-glutamine. The cells were cultured in an incubator at 37°C infused with 5% CO_2.

Cells of every cell-line were seeded at a density of 20,000 cells per well on a 24-well plate. Four wells each were infected at a multiplicity of infection (MOI) of 0.001 with FeLV-A/Glasgow-1 or FeLV-A/261*env* supernatant. Two wells contained uninfected cells as negative control. Infection time was four hours, followed by a washing step with RPMI. Cells were passaged, when reaching a confluency of 90% and maintained up to thirteen days. FeLV p27 ELISA was performed at days 3, 5, 8, 10 and 13 to detect a productive infection.

4.8 PCR assays

4.8.1 Detection of FeLV subgroups by conventional PCR

FeLV-subgroups were investigated by conventional PCR using the FeLV-A specific primers RB59 and RB17, the FeLV-B specific primers RB53 and RB17 and the FeLV-C specific primers RB58 and RB47 as described (Mathes et al., 1994, Sheets et al., 1993). (Table 2)

Table 2: Primer for FeLV subgroup PCR

FeLV-subgroups	Primer Name	Sequence 5'-3'	Reference
FeLV-A	RB59	CAATGTAAAACACGGGGCAC	
FeLV-A / FeLV-B	RB17	TAGTGATATTGGTTCTCTTCG	(Mathes et al.,
FeLV-B	RB53	ACAACGGGAGCTAGTG	1994, Sheets et al.,
FeLV-C	RB58	AGATCTTGGGCACGTTATTCC	1993)
	RB47	TTGTGAAATGGCATTGCTGC	

4.8.2 *Env* TaqMan® and S261-III *env* real-time PCR assays

GenomeWalker libraries were tested with the *env* TaqMan® real-time PCR which is specific for FeLV-A/Glasgow-1 and the SP261-III *env* PCR that is specific for the Variant S261-III (Helfer-Hungerbuehler et al., 2010, Flynn et al., 2002) to determine the ratio of S261-III to FeLV-A/Glasgow-1. From every library one sample was tested in both PCRs. The mean for the *env* TaqMan® real-time PCR was 1.5 (range 0 – 5.4) copies per reaction while the mean of the S261-III *env* PCR was 52,000 (range 49,300 – 57,100) copies per reaction, which is roughly 1 copy of FeLV-A/Glasgow-1 to 35,000 copies of variant S261-III. For details to the used PCR systems see Table 3.

Table 3: Primer and Probes for FeLV real-time PCR assays

System	Name	Sequence 5'-3'	Reference
Env specific Glasgow-1	FeLVenvf	GCCCCAAACGAATGAAAGC	(Flynn et al., 2002)
	FeLVenvr	AATCCGTTTGGGACCCATG	
	FeLVenvp	CCCAAGGTCTGTTGCCCCCACC[1]	
SP261-III env PCR	261-gen-F-CORRECT	GATCCGGACCGACCATAATTAA	(Helfer-Hungerbuehler et al., 2010)
	Primer 261gen-IIIR	ACACCACTGCAGTAGCTGGCTAA	
	261genP	TGTATGATTCCATTTAGTCCC[1]	

[1] 5' FAM/ 3' TAMRA

4.8.3 FeLV *gag* TaqMan® real-time PCR assay

A new real-time PCR assay was designed in the highly conserved *gag* region for better assessment and comparison of the different *env* and LTR variants. Probe and primer sequences were designed using Geneious software (version 8.1, Biomatters Limited). Primer and probe sequences were selected within the *gag* region of FeLV-A/Glasgow-1, to ensure that all recombinant provirus variants were detectable, but not endogenous FeLV sequences; this was confirmed using genomic TNA from 30 FeLV-negative SPF cats (data not shown). PCR amplification using the forward primer G1-1515F (5'-CAACAACCGACCCCAGTATT-3') in a concentration of 0.3 µM and the reverse primer G1-1611R (5'-AGTTAGGGCCACTGGATCTT-3') in a concentration of 0.9 µM resulted in a PCR product of 97 bp. The probe (5'-CAGCTTCAGACTTGTATAACTGGAAGTCGCA-3') was labelled at the 5'-end with the fluorescent reporter dye FAM and at the 3'-end with the dark quencher dye BHQ-1. DNA was amplified and quantified in an ABI Prism 7500Fast sequence detection system using TaqMan® Fast Universal PCR Master Mix (Applied Biosystems). Optimal PCR conditions are as follows: One cycle of 95°C for two min, 45 cycles of 94°C for 3 s followed by 60°C for 30 s.

For the production of a standard for absolute quantification a sequence in the FeLV *gag* region with the length of 515 bp was cloned into pUC57-Kan (GeneScript USA Inc., Piscataway, New Jersey) using *Eco*RV as cloning site. The plasmid was digested with *Hind*III (Thermo Scientific). Ten-fold serial dilutions of the standard with nuclease-free water containing 30 µg/ml carrier salmon sperm DNA (Invitrogen, Basel, Switzerland) was made starting from 10^8 copies per 5 µL to 10^{-1} copies per 5

µL. The real-time TaqMan® PCR system was optimized as described in (Kessler et al., 2009) using cDNA and a 3 × 3 primer matrix with 50, 300 and 900 nM end concentrations. Each of the nine conditions was run in quadruplicate under the conditions described above. Moreover, using the best primer concentration, five different probe end concentrations (50, 100, 150, 200 and 250 nM) were tested for optimal performance. The sensitivity of the *gag* real-time PCR assay was determined by an endpoint dilution experiment: Triplicates of standard dilutions from 10^8 to 10^2 copies per reaction and ten replicates of dilutions from 10^1 to 10^{-1} copies per reaction were tested. The sensitivity of the assay is given by the dilution in which at least seven of 10 replicates are still positive (Lockey et al., 1998).
The intra-run precision of the TaqMan® real-time PCR assay was determined using tenfold serial dilutions of the plasmid standard. Between-run precision was assed using the 10^5 dilution in five separate experiments. For all measurements, mean value, standard deviation and coefficients of variation (CV) were calculated for the threshold cycle (C_T).

4.9 Statistics

Statistical analyses were performed with GraphPad Prism for Windows (version 3.0, GraphPad software, San Diego, CA). Differences among three or more groups were analyzed by the non-parametric Kruskal-Wallis one-way ANOVA by Ranks (p_{KW}) for unpaired samples and Dunn's posttest. If only two groups were compared, the Mann–Whitney U-test (p_{MWU}) was used. Differences were considered significant if $p < 0.05$.

5 Results

5.1 Evaluation of the newly developed *gag* TaqMan® real-time PCR assay

A *gag* TaqMan® real-time PCR was developed in a conserved area to detect all FeLV variants. Primer and probe concentrations for the newly designed *gag* TaqMan® real-time PCR assay were optimized using cDNA of an experimentally FeLV-A/Glasgow-1 infected cat and a primer matrix and different probe concentrations. The maximal sensitivity (minimal Ct values) and very high fluorescence was reached using the concentrations listed in Table 4.

Table 4: Optimal final concentration of primers and probe for the *gag* TaqMan® real-time PCR

Forward Primer (nM)	**Reverse Primer (nM)**	**Probe (nM)**
300	900	250

Using these concentrations, the amplification efficiency of the new *gag* TaqMan® real-time PCR assay was >96%. The lower detection limit of the assay was equal to one copy of target standard plasmid per reaction in an endpoint dilution experiment (10 out of 10 reactions with a single copy/reaction were positive). We observed linearity of the assay over a 10^8-fold range. The coefficient of variation for the intra-run precision CV_{intra} was 0.53% ± 0.32 and for the inter-run precision CV_{inter} 0.65% ± 0.57.

5.2 Proviral integration sites of FeLV-A/Glasgow-1 progeny viruses

GenomeWalker libraries were tested with the exo FeLV U3 (Tandon et al., 2005) and *env* TaqMan® real-time PCR (Helfer-Hungerbuehler et al., 2010, Flynn et al., 2002) to determine the variant SP261-III to FeLV-A/Glasgow-1 ratio, which was up to 35,000 copies of the variant SP261-III versus one copy of FeLV-A/Glasgow-1. Using genome walking we screened proviral integration sites in tumor tissues of animals #261 (sternal lymph node, LN) and #67 (tumor tissue of the thymic lymphoma, T). The sequences were blasted and mapped on the cat reference genome; 12 out of 33 blasted sequences could be placed on the cat genome; the remaining 21 sequences were vector sequences. Out of the 12 feline sequences, eight derived from cat #261 and four sequences were from cat #67. Sequences from the cat #261 were mapped on five different chromosomes and those from cat #67 on two different chromosomes, including two different locations on chromosome D4 (Table 5). In four cases two independent inserts in the same region could be found. However, none of the insertion sites were identical in cats #261 and #67.

The integration sites found in cats #261 and #67 were compared to previously described common insertion sites (CIS) in the cat (myc, pim-1, flvi-1, flvi-2 or bmi-1, flit-1, fit-1) (Fujino et al., 2009, Tsatsanis et al., 1994, Levy et al., 1993). None of the herein found insertions sites were identical with previously described CIS in cats and the novel insertion sites were all located on different chromosomes then the previously described CIS in cats (which were on B2, B4 and F2).

While most of the integrations occurred in regions between host genes, two inserts were found within two genes in cat #261. These two genes were the Pseudouridylate Synthase 10 (*PUS10;* in intron) and the Sorting Nexin 25 (*SNX25;* in intron and exon). For cat #261 other integrations occurred between MAP3K12 binding inhibitory protein 1 (*MBIP*) and thyroid transcription factor 1 (*TTF1* or *NKX2*-1), between the genes dopamine receptor D2 (*DRD2*) and transmembrane protease, serine 5 (*TMPRSS5*) and between the Toll-like receptor 5 (*TLR5*) and the sushi domain containing 4 (*SUSD4*) genes. For cat #67 the integrations occurred between growth factor independent 1B transcription repressor (*GFI1B*) and general transcription factor IIIC, polypeptide 5 (*GTF3C5*), between the genes family with sequence similarity 102, member A (*FAM102A*) and nuclear apoptosis inducing factor 1 (*NAIF1*) and between the NK2 Homeobox 2 (*NKX2*-2) and the paired box 1 (*PAX1*) genes.

Table 5: Listed are the integration sites found in cat #261 and cat #67. The 5' and 3' genes, their functions (selected) and the distance to the proviral integration site are listed according to the feline genome. Potentially tumor associated functions in bold.

Cat (Tissue)	**Cat chrom.**	**Gene 5'**	**Functions**	**Distance***	**No. of inserts**	**Distance**	**Gene 3'**	**Functions**	**Hum. chrom.**
261 (LN)	A3	*PUS10*	Apoptosis	In intron 3	**2**	In intron 3	*PUS10*		2p16.1
	B1	*SNX25*	Function in cellular trafficking	In intron 6 In exon 7	**2**	In intron 6 In exon 7	*SNX25*		4q35.1
	B3	*MBIP*	Protein kinase inhibitor, chromatin organization	79.0 kb	**2**	87.0 kb	***NKX2-1***	**Tumor associated**, oncogene or tumor suppressor	14.q13.3
	D1	***DRD2***	D2 subtype of the dopamine receptor, **upregulated in some cancers**	58.0 kb	1	117.0 kb	*TMPRSS5*	Protease of the serine protease family	11q23.2
	F1	*TLR5*	Pathogen recognition, NF-kB mobilization	18.0 kb	1	10.0 kb	*SUSD4*	Complement inhibitor	1q41
67 (T)	D4	***GFI1B***	Homolog to GFI1 = **integration site of MLV-induced lymphoma in rats**	8.0 kb	1	10.0 kb	*GTF3C5*	RNA polymerase III-mediated transcription	9q34.13
	D4	*FAM102A*	Estrogen action and bone homeostasis	2.3 kb	**2**	48.0 kb	***NAIF1***	**Apoptosis, down-regulated in cancer**	9q34.11
	A3	***NKX2-2***	Target gene and **tumor marker** in Ewing's sarcoma	29.0 kb	1	133.0 kb	***PAX1***	**Tumor suppressor, silenced in cancer**	20p11.22

LN: Lymphoma sternal lymph node. T: Thymic lymphoma; for gene abbreviations see text. MLV: Murine leukemia virus. * If the insertion site was within the gene, the intron or exon number is listed.

During the application of the GenomeWalker kit questions were raised whether the provided restriction sites were viable using in the cat genome and whether some integration sites would be missed if restriction sites were absent from genes or too far from each other. To exclude a bias from the chosen restriction enzymes, the restriction sites present in the two genes found in cat #261 (*PUS10* and *SNX25*) were examined with the Geneious program and compared to the restriction sites of three CIS (*myc, pim-1, bmi-1*) described in the cat (see Table 6). A maximum deviation of 2.5-fold was seen in the restriction analysis of the enzyme *Dra*I between *PUS10* and *myc*. The total count of restriction sites over the 500 kb length of each gene is similar, as one could theoretically find a restriction site every 470 bp (for *PUS10* with the maximal count of sites) or 640 bp (for *myc* with the minimal count of sites), respectively. The numbers show that for example the detection of a supposed integration near myc would have been possible with the chosen restriction sites.

Table 6: Restriction enzyme digestion of 500 kb surrounding the genes (250 kb 3' and 5' of the gene of interest) found in cat #261 (*PUS10*, *SNX25*) compared with three different CIS genes (*myc, pim-1, bmi-1*) with the restriction enzymes used in the GenomeWalker 2.0 kit.

Gene	***Dra*I**	***Eco*RV**	***Pvu*II**	***Stu*I**	**Total**
PUS10	859	64	152	82	1157
SNX25	795	83	185	88	1151
myc	312	49	262	166	793
pim-1	499	53	220	149	921
bmi-1 (Flvi-2)	716	50	161	115	1042

5.3 Characterization of the recombinant provirus constructs

5.3.1 Sequence comparison of the *env* variants

Three heavily mutated FeLV-A *env* variants were identified previously (Helfer-Hungerbuehler et al., 2010). For the current study the most abundant *env* variant, SP261-III [GeneBank: EU359305], was used and cloned into the backbone of FeLV-A/Glasgow-1 and further characterized in an *in vitro* experiment (see chapter 5.4 and 5.5). In Figure 4 an alignment of the amino acid sequences of the FeLV envelope of FeLV-A/Glasgow-1 and SP261III is shown. The amino acid identity was 91%. Sequence divergences between these two *envs* were pronounced at the regions variable region A (VRA), variable region B (VRB), variable region C (VRC), proline-rich region (PRR) and the C2-loop.

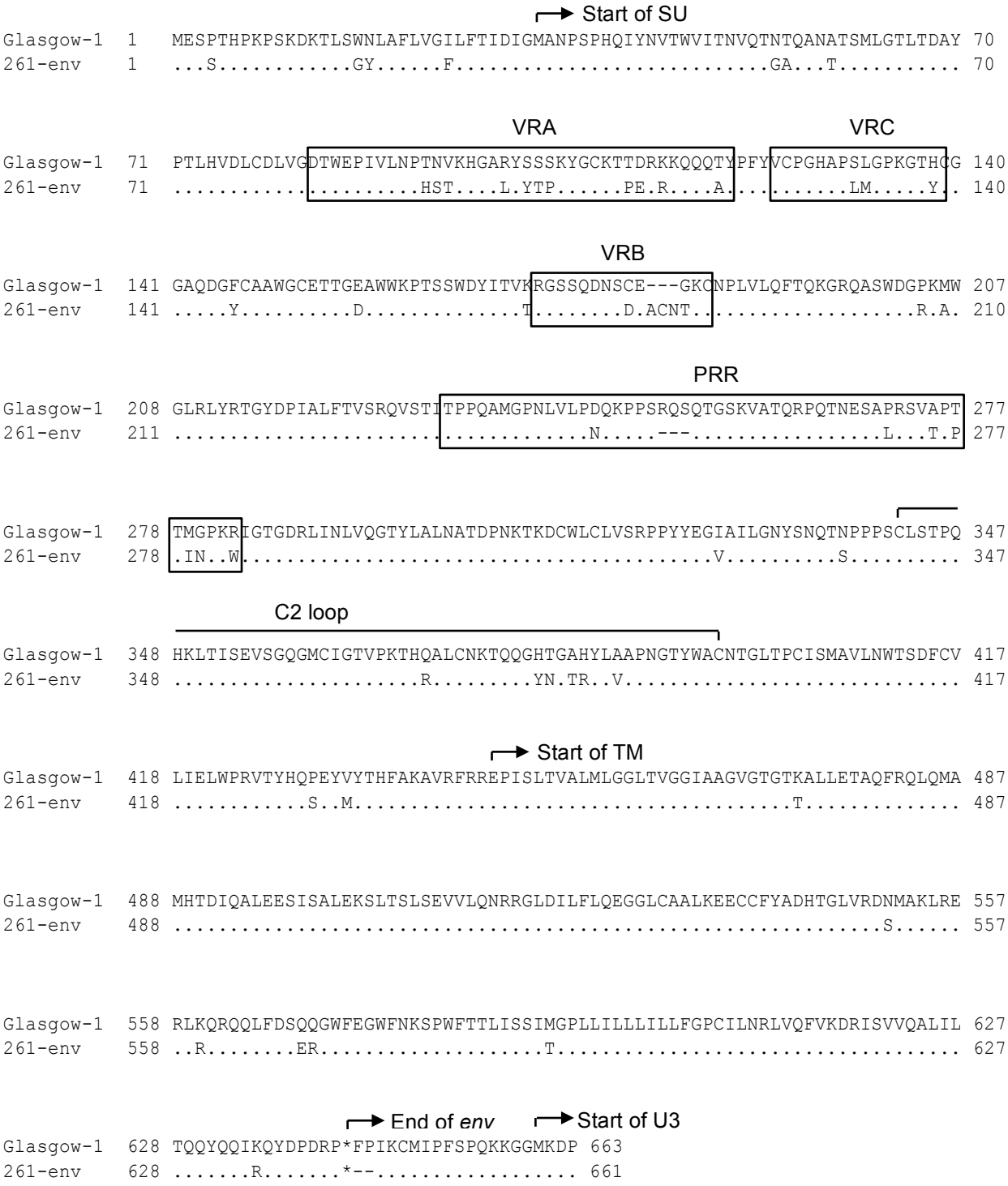
Start of SU
VRA
VRC
VRB
PRR
C2 loop
Start of TM
End of env
Start of U3

Figure 4: Amino acid alignment of FeLV *env* sequences. Amino acid alignment of the *env* coding region from FeLV-A/Glasgow-1 [GeneBank: M12500] and the variant SP261-III (261*env*) [GeneBank: EU359305]. The start of the SU region, the transmembrane domain (TM), the variable regions VRA, VRB and VRC, the PRR and the C2 disulfide-bonded loop (S-S) are indicated (according to (Rey et al., 2008b)). Dots represent identical residues, and dashes represent spaces, which were introduced for proper alignment. Adapted from (Rey et al., 2008b).

5.3.2 Sequence comparison of the LTR variants

Previously nine different LTR variants had been described (Helfer-Hungerbuehler et al., 2010). Out of these nine variants two were available for this study (261L11 and 261L12 [GenBank: FJ613291 and FJ613295]). In addition, two new variants were found and included into the study (261L1 and 261L3 [GenBank: KU962189 and KU962190]). Four LTR-recombinants were constructed. An overall sequence conservation of 96-98% was seen when the full-length progeny LTR sequences were compared to FeLV-A/Glasgow-1 (see Table 7).

Table 7: Genetic distances between LTRs of FeLV-A/Glasgow-1 and of FeLV-A/261 recombinants

	Glasgow-1	**261L1**	**261L3**	**261L11**	**261L12**
Glasgow-1					
261L1	96.48%				
261L3	97.10%	97.31%			
261L11	98.34%	97.31%	97.52%		
261L12	96.49%	94.22%	96.90%	96.07%	

The alignment of the four different FeLV-A/261 LTR variants and FeLV-A/Glasgow-1 showed 33 locations of point mutations, insertions or deletions (see Figure 5). Changes were found in the simian virus 40 core enhancer (CORE), the Leukemia virus factor b (LVb), the nuclear factor 1 (NF1), the glucocorticoid response element (GRE) and the FeLV-specific binding motif (FLV-1). 261L1 and 261L12 had changes in the LVb, the CORE, the NF1 and the GRE sites. 261L3 showed changes in the NF1 and the 261L11 had changes in the NF1. Influence of these mutations on replication efficiency and host range was investigated in further studies (see 5.4 and 5.5).

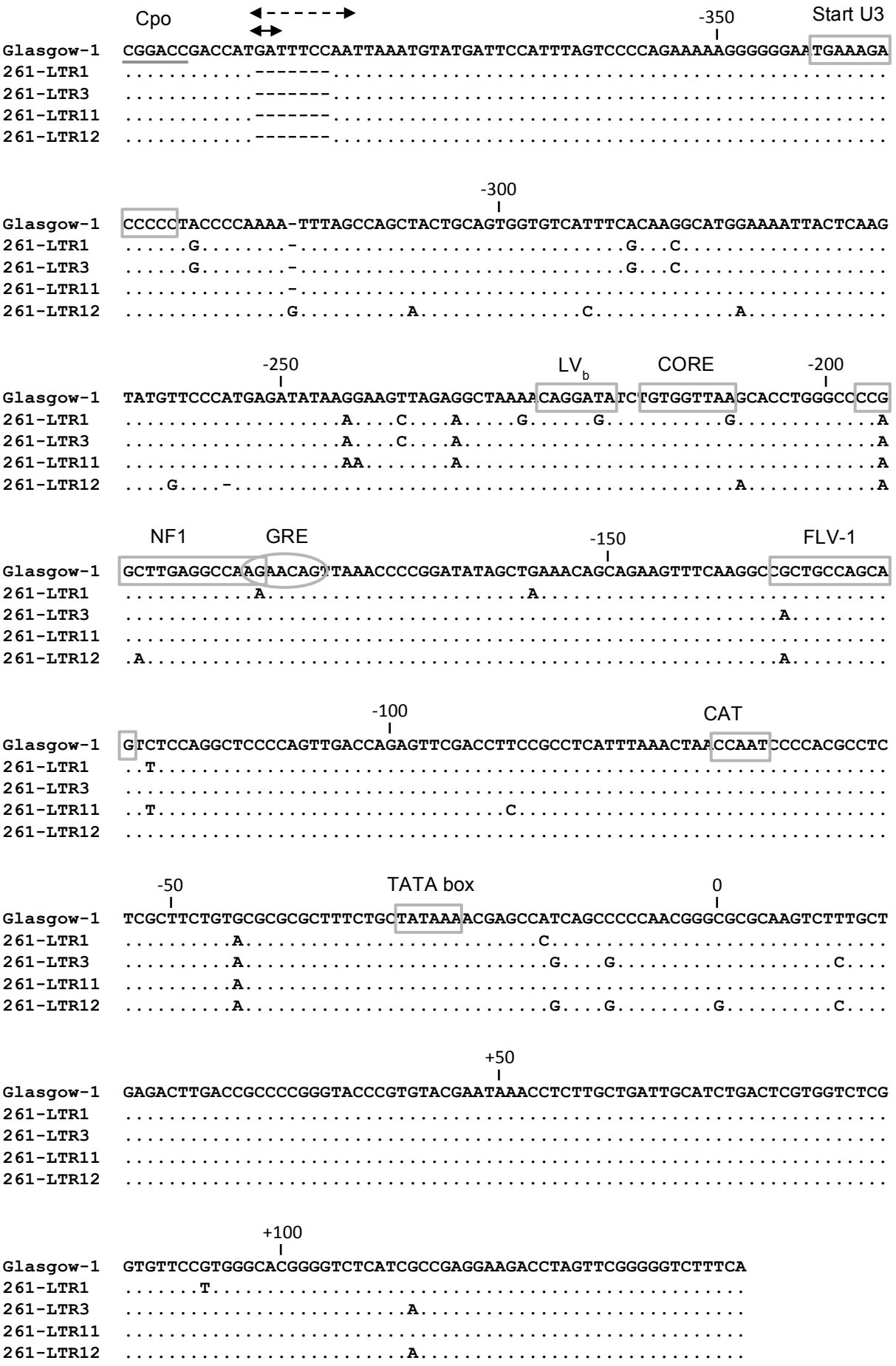
Cpo
-350
Start U3
Glasgow-1 CGGACCGACCATGATTTCCAATTAAATGTATGATTCCATTTAGTCCCCAGAAAAAGGGGGGAATGAAAGA
261-LTR1 .....-------.....
261-LTR3 .....-------.....
261-LTR11 .....-------.....
261-LTR12 .....-------.....
-300
Glasgow-1 CCCCCTACCCCAAAA-TTTAGCCAGCTACTGCAGTGGTGTCATTTCACAAGGCATGGAAAATTACTCAAG
261-LTR1 ......G.....-.....G...C.....
261-LTR3 ......G.....-.....G...C.....
261-LTR11 .....-.....
261-LTR12 .....G.....A.....C.....A.....
-250
LV b
CORE
-200
Glasgow-1 TATGTTCCCATGAGATATAAGGAAGTTAGAGGCTAAAACAGGATATCTGTGGTTAAGCACCTGGGCCCCG
261-LTR1 .....A....C....A.....G.....G.....G.....A
261-LTR3 .....A....C....A.....A
261-LTR11 .....AA.....A.....A
261-LTR12 ....G....-.....A.....A
NF1
GRE
-150
FLV-1
Glasgow-1 GCTTGAGGCCAAGAACAGTTAAACCCCGGATATAGCTGAAACAGCAGAAGTTTCAAGGCCGCTGCCAGCA
261-LTR1 .....A.....A.....
261-LTR3 .....A.....
261-LTR11 .....
261-LTR12 .A.....A.....
-100
CAT
Glasgow-1 GTCTCCAGGCTCCCCAGTTGACCAGAGTTCGACCTTCCGCCTCATTTAAACTAACCAATCCCCACGCCTC
261-LTR1 ..T.....
261-LTR3 .....
261-LTR11 ..T.....C.....
261-LTR12 .....
-50
TATA box
0
Glasgow-1 TCGCTTCTGTGCGCGCGCTTTCTGCTATAAAACGAGCCATCAGCCCCCAACGGGCGCGCAAGTCTTTGCT
261-LTR1 .....A.....C.....
261-LTR3 .....A.....G....G.....C....
261-LTR11 .....A.....
261-LTR12 .....A.....G....G.....G.....C....
+50
Glasgow-1 GAGACTTGACCGCCCCGGGTACCCGTGTACGAATAAACCTCTTGCTGATTGCATCTGACTCGTGGTCTCG
261-LTR1 .....
261-LTR3 .....
261-LTR11 .....
261-LTR12 .....
+100
Glasgow-1 GTGTTCCGTGGGCACGGGGTCTCATCGCCGAGGAAGACCTAGTTCGGGGGTCTTTCA
261-LTR1 .....T.....
261-LTR3 .....A.....
261-LTR11 .....
261-LTR12 .....A.....

Figure 5: Comparison of nucleotide sequences of the FeLV U3 region. Nucleotide comparison of the U3 region from four LTR variants retrieved from cat #261 and FeLV-A/Glasgow-1 [GenBank: M12500]. Nuclear protein-binding sites (LV_b, CORE, NF1; GRE and FLV-1) and their corresponding nucleotide sequences are marked in the reference strain FeLV-A/Glasgow-1 (according to (Hisasue et al., 2009)). Dots indicate identity with FeLV-A/Glasgow-1 sequence. Dashes indicate gaps. The stop codon of *env* in FeLV-A/Glasgow-1 is indicated with a solid left right arrow (TGA) the one of the variants with a dashed left right arrow (TAA). Nucleotides differing from the originally inoculated strain are indicated. Numbers at the top of the sequence indicate nucleotide positions relative to the presumptive RNA cap site in the FeLV-A/Glasgow-1 LTR (Stewart et al., 1986).

5.4 Replication efficiency of FeLV-A/Glasgow-1 and progeny viruses

In vitro experiments were performed to determine whether the mutational changes in the FeLV-A/261 *env* and LTR recombinants influenced the rate of virus replication. Plasmid DNAs encoding infectious viruses of the recombinants and the original challenge strain FeLV-A/Glasgow-1 (pFGA-5) were transfected into FEA cells. Virus replication was then quantified by measuring FeLV p27 ELISA antigen levels and viral RNA using quantitative real-time *gag* PCR from cell culture supernatants at regular time intervals (Figure 6A-J).

Significant differences in replication efficiency were found between the original challenge strain FeLV-A/Glasgow-1 and the recombinants containing *env* and LTR of the progeny viruses. Remarkably, the progeny *env* and LTR recombinants showed largely lower replication efficiency than the prototype virus FeLV-A/Glasgow-1 (Figure 6 and Figure 7). The differences were more pronounced, when p27 antigen was measured in the cell culture supernatant then when viral RNA was quantified.

A constant significant difference over the whole 24-days observation period was found for FeLV-A/261*env* starting at day 3 after transfection using p27 antigen detection and at day 5 after transfection using viral RNA as read-out in the cell culture supernatant (Figure 6E and J and Figure 7, $p_{MWU} < 0.05$). Similarly, a constantly lower replication efficiency was found for variants 261L1 and 261L11 in the p27 antigen ELISA (Figure 6F and H; $p_{MWU} = 0.0286$). Moreover, variant 261L12 was also constantly lower when measuring viral RNA loads (Figure 6D; $p_{MWU} = 0.0286$) and with the exceptions of days 5 and 10 in p27 antigen loads (Figure 6I; $p_{MWU} = 0.0286$). In contrast, the variant 261L3 only had a poor replication rate during the initial phase of virus replication (10 days for viral RNA and 17 days for p27 antigen levels) but then reached similar viral RNA levels like the prototype virus FeLV-A/Glasgow-1 (Figure 6B and G).

In order to confirm that this change over time in replication efficiency of all variants was not due to mutational changes in the LTR acquired during the in vitro experiment, the variants were re-sequenced at the end of the replication study. No changes in the LTRs were observed.

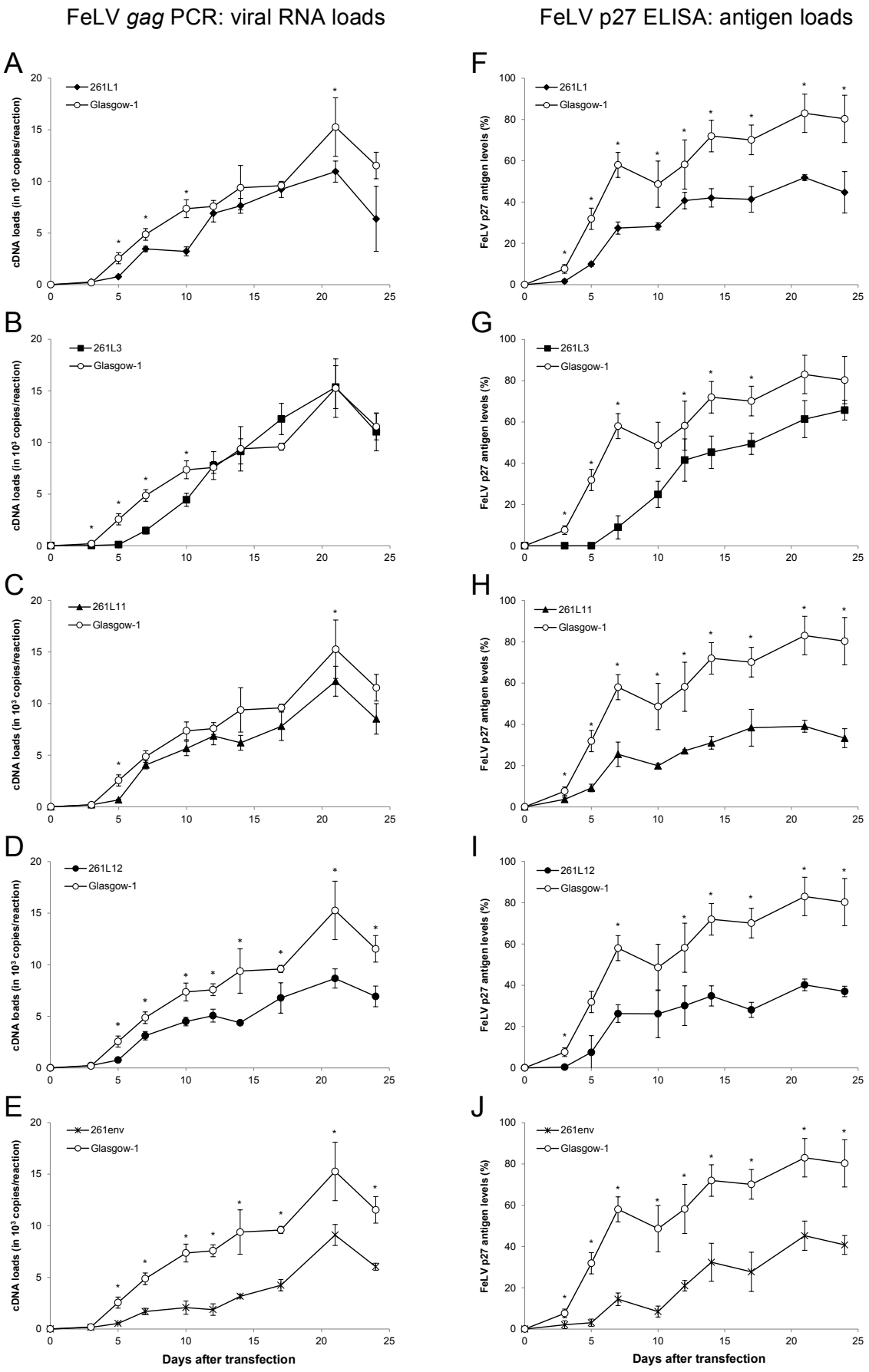
FeLV *gag* PCR: viral RNA loads
FeLV p27 ELISA: antigen loads
A
B
C
D
E
F
G
H
I
J
261L1
261L3
261L11
261L12
261env
Glasgow-1
cDNA loads (in 10³ copies/reaction)
FeLV p27 antigen levels (%)
Days after transfection

Figure 6: Results of FeLV *gag* PCR (A-E) and p27 ELISA (F-J) in a replication efficiency experiment comparing each progeny virus variant against FeLV-A/Glasgow-1 during the time course of 24 days. Each data point represents the mean value and its standard deviation of four replicates. In A and F data of the *env*-variant compared to FeLV-A/Glasgow-1 are shown. B-E and G-J contain data of the LTR-variants compared to FeLV-A/Glasgow-1. Differences between the variants against FeLV-A/Glasgow-1 (pFGA-5) were tested for significances using the Mann-Whitney U-test: * = $p < 0.05$.

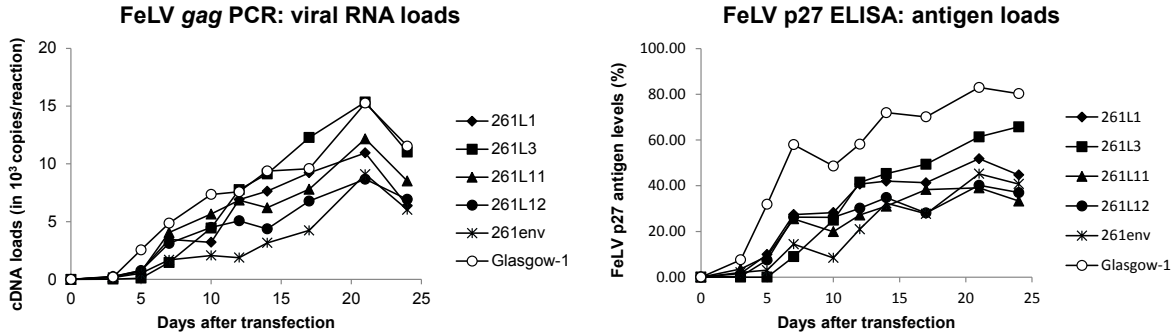

Figure 7: Summary of the results of FeLV *gag* PCR (left) and p27 ELISA (right) in a replication efficiency experiment comparing each progeny virus variant against FeLV-A/Glasgow-1 during the time course of 24 days. Each data point represents the mean value of four replicates.

5.5 Titration and host range of FeLV-A/Glasgow-1 and progeny viruses

5.5.1 Results of the titration experiment

The titration experiment was carried out to determine infectivity (multiplicity of infection or MOI) of the viruses FeLV-A/Glasgow-1 (Table 8) and FeLV-A/261*env* (see Table 9) in FEA cells. The results from these experiments were used to determine dilutions with similar infectivity for comparing both virus strains in the later host range experiment. The MOI, *M*, is given by the equitation $M = (0.7 \times N \times V)/(Df \times X)$, where *N* is the $TCID_{50}$ calculated by the Spearman & Kärber algorithm, *V* is the volume of virus stock per Well, *Df* is the dilution factor and *X* is the amount of cells per well. In Table 10 the values used in the calculations and their results for FeLV-A/Glasgow-1 and FeLV-A/261*env* are shown.

For the later host range experiment we used the undiluted stock solution of FeLV-A/Glasgow-1 and a tenfold dilution thereof to match the virus concentration of the undiluted stock solution of FeLV-A/261*env*.

Table 8: Results of FeLV p27 ELISA in percentage at day 6 after infection in the titration experiment testing FeLV-A/Glasgow-1 on FEA cells. Every result > 4% was rated positive and indicated in bold.

Stock	**10^{-1}**	**10^{-2}**	**10^{-3}**	**Negative control**
36.2	**13.3**	**6.4**	0.0	2.4
26.0	**11.1**	3.9	0.8	3.2
29.0	**11.9**	1.1	0.3	2.4
27.7	**14.2**	**4.8**	0.8	3.0

Table 9: Results of FeLV p27 ELISA in percentage at day 8 after infection in the titration experiment testing FeLV-A/261*env* on FEA cells. Every result > 4% was rated positive and indicated in bold.

Stock	**10^{-1}**	**10^{-2}**	**10^{-3}**	**Negative control**
11.2	1.3	0.0	0.0	0.2
15.5	3.8	0.0	0.5	0.0
13.5	2.7	0.0	0.1	0.0
9.9	0.0	0.2	0.0	0.0

Table 10: Listed are the values used in the equitation $M = (0.7 \times N \times V)/(Df \times X)$ to calculate the MOI for FeLV-A/Glasgow-1 and FeLV-A/261*env*.

Variable	**Meaning**	**FeLV-A/Glasgow-1**	**FeLV-A/261*env***
N	TCID50/ml	100	3.16
V	Volume / Well	1 ml	1 ml
X	Cells / Well	20,000	20,000
Df	Dilution factor	1	1
M	**MOI**	**0.0035**	**0.00011**

5.5.2 Results of the host range experiment

The host range is determined by the receptor usage that is specific for each FeLV subgroup. Mutational changes in regions that are crucial for receptor interactions and viral entry might change the host range.

The two feline cell lines, FEA and CRFK, were successfully infected not only by FeLV-A/Glasgow-1 but also by FeLV-A/261*env* (Table 11). HeLa, *M. dunni*, ST-IOWA and 104C1 were not susceptible to an infection with either FeLV-A/Glasgow-1 or FeLV-A/*261env*. In contrast canine MDCK cells could only be infected with FeLV-A/Glasgow-1 but not with FeLV-A/261*env*. While HEK293 cells should be susceptible to an infection with FeLV-A (Nakata et al., 2003) only the higher virus concentration (MOI 0.0035) of FeLV-A/Glasgow-1 led to an infection. No infection of HEK293 cells was observed by FeLV-A/Glasgow-1 at a lower MOI of 0.00035 and by FeLV-A/261*env* at the MOI of 0.00011.

Table 11: Host Range of FeLV-A/Glasgow-1 and FeLV-A/261*env*

Species	**Cell line**	**Glasgow-1 (MOI 0.0035)**	**Glasgow-1 (MOI 0.00035)**	**FeLV-A/261*env* (MOI 0.00011)**
Cat	FEA	++/+++	+/++	+/++
	CRFK	**+/++**	nt	**+++**
Human	HeLa	-	-	-
	HEK293	++	-	-
Pig	ST-IOWA	-	-	-
Guinea Pig	104C1	-	-	-
Mouse	*M. dunni*	-	-	-
Dog	MDCK	+++	**+/++**	-

FeLV p27 ELISA: + > 4%; ++ > 10%; +++ > 20%; nt = not tested

6 Discussion

In the present study we have characterized highly divergent FeLV-A/Glasgow-1 variants from the experimentally FeLV-infected cat #261 by examining the viruses' insertion sites, replication efficiency and host range. The FeLV variants arose during a long-term (8.5 years) FeLV-FIV-infection and were of interest due to their high degree of mutations in the *env* gene (89-92% amino acid identity of the variants to FeLV-A/Glasgow-1) and LTR (96-98% amino acid identity of the variants to FeLV-A/Glasgow-1). In addition, the virus loads of the progeny variants were significantly higher *in vivo* compared to the original inoculation strain (Helfer-Hungerbuehler et al., 2010).

6.1 Integration sites

The occurrence of malignant diseases in FeLV infection occurs over a time course of several weeks to a few years after virus exposure (Hoover and Mullins, 1991, Hartmann, 2011, Hofmann-Lehmann et al., 1997, Tsatsanis et al., 1994, Beatty, 2014). FeLV usually does not carry oncogenes; rather insertional mutagenesis has been identified as a crucial factor in the development of lymphomas. The onset of those malignancies is associated with several mechanisms of insertional mutagenesis. One of these mechanisms is insertional activation of oncogenes at the sites of proviral integration either by replacing the endogenous promoter with the very active promoter contained in the 3' LTR U3 region or by enhancing endogenous promoter activation by the enhancer element also part of the same U3 region (see also Figure 1 and Figure 5). This oncogenic potential may also affect genes at greater distance up to 300 kb up and down stream of the integration site (Lazo et al., 1990, Johnson et al., 2005, Kool and Berns, 2009) and is even greater when tandem repeats in the 3' LTR U3 are found (Chandhasin et al., 2005b). Although no enhancer repeats were detected in the LTR variants of cat #261, several mutations in the enhancer region were identified. Thus, characterization of these variants and their integration sites might provide further insight into viral enhancer mechanisms. Another mechanism works by disrupting genes by proviral insertion; this is especially the case, if tumor suppressor genes are affected. Because of these mechanisms, analysis of insertion sites has been useful as a tool to identify genes, which are associated with malignancies.

In the present study we aimed to identify genes involved in FeLV-A/Glasgow-1-induced lymphomas in two experimentally infected cats, cat #261 and cat #67, by performing integration site analysis using genome walking. Both cats were kept under similar barrier conditions. As previously described the cat #261 was a special case as it was co-infected with FeLV and FIV and had a B-cell lymphoma. Therefore, we also analyzed the insertion sites in cat #67 that developed a FeLV-A associated T-cell lymphoma after three years of viremia; the latter is an infection outcome we have observed in several FeLV-A/Glasgow-1 mono-infected cats (Hofmann-Lehmann et al., 1995, Helfer-Hungerbuehler et al., 2015).
Interestingly, the integration sites found in the present study in the two FeLV-A/Glasgow-1 infected cats were all distinct from common integration sites previously described in FeLV-infected cats (Table 5). Three different databases (RTCGD, TAG, COSMIC) were queried to assess whether the genes close to the integration sites had already been described in tumorigenic processes in humans or mice. From the list of identified insertion sites in cat #261 and #67, there were some insertions near genes with oncogenic potential (NKX2-1, NKX2-2, DRD2) and genes involved in apoptosis (PUS10, NAIF1) or with repressor function (GFI1B, PAX1).

In the following all genes within proximity of the detected integration sites and their function in humans and mice are discussed beginning with the ones determined in cat #261 followed by those in cat #67. For each cat, the genes are listed and reviewed according to their potential importance in tumorigenic processes according to the literature and our opinion.
In cat #261 we found integrations in-between genes (*NKX-2-1* and *MBIP*, *DRD2* and *TMPRSS5*, *TLR5* and *SUSD4*) and integrations within two genes (*PUS10* and *SNX25*).
NKX2-1 is also known as thyroid transcription factor 1 (*TTF-1*). In one of the queried databases it showed up as a tumor associated gene and is known as a molecular marker in human lung cancer and useful in identifying the origin tissue of metastases (Holzinger et al., 1996). The role of *NKX2-1* in cancer pathogenesis is very complex, because it can act as both, a tumor suppressor or oncogene (Mu, 2013). An overexpression of NKX2-1 is found in about 12% of cases of human lung adenocarcinoma (Weir et al., 2007), but low *NKX2-1* expressions in those tumors are generally associated with poor prognosis (Barletta et al., 2009). In addition, in a study

using a mouse model of lung adenocarcinoma, a loss of mouse *NKX2-1* promoted metastasis (Winslow et al., 2011). ***MBIP*** is located right next to *NKX2-1* and has also been mentioned in association with an increased thyroid cancer risk; however, the later may also be due to its proximity to *NKX2-1* (Porcu et al., 2013). *MBIP* is involved in chromatin organization and is a protein kinase inhibitor.

DRD2 encodes the D2 subtype of the dopamine receptor. It was found, that DRD2 is upregulated in some cancers of the pituitary gland (Wood et al., 1991) and of the gastrointestinal tract (Mu et al., 2017). In one study an upregulation of DRD2 in human pancreatic cancer was observed and it was reported that treating xenograft mice with DRD2 antagonist reduced proliferation and migration of tumor cells (Jandaghi et al., 2016).

TLR5 plays a role in pathogen recognition, specifically the flagellin of bacteria and mobilizes the nuclear factor NF-kB (Smith and Ozinsky, 2002, Zeng et al., 2006). NF-kB has ambiguous effects concerning cancer development and treatment. An activation of NF-kB might be beneficial in fighting cancer, because it enhances the expression of genes, which are helping in attracting and activating neutrophils, natural killer (NK) cells and dendritic cells. These cells play important roles in immune responses against pathogens and tumor cells (Yang et al., 2016). Thus, toll like receptor agonists might have a potential as therapeutics for cancer (Connolly and O'Neill, 2012). But there are also adverse effects by activating NF-kB in tumor cells. NF-kB helps in recruiting pro-inflammatory cytokines, thus creating a tumorigenic microenvironment. Additionally, NF-kB activation in tumor cells stimulates cell proliferation, prevents apoptosis, regulates tumor angiogenesis, promotes tumor metastasis and even has influence on the energy metabolisms of tumor cells (Xia et al., 2014). It would be interesting to find out whether an overexpression of *TLR5* caused by the enhancer of FeLV-U3 region would lead to a pro-tumorigenic environment or aggravate already existing tumorigenic diseases.

SUSD4 was shown to function as a complement inhibitor and was also found activated in tumor infiltrating cells in different cancers (Holmquist et al., 2013). Complement activation can either kill cancer cells or create an inflammatory environment, which promotes cancer progression (Donin et al., 2003, Markiewski et al., 2008), thus the role of *SUSD4* in cancer is not yet fully understood. One study showed that an expression of *SUSD4* was associated with a better prognosis for patients suffering from breast cancer (Englund et al., 2015).

PUS10 is involved in apoptosis as it has been identified as a modulator of TRAIL-induced cell death using RNA interference (RNAi) library screening (Aza-Blanc et al., 2003). TRAIL (TNF-related apoptosis-inducing ligand) is a cytokine, which binds to the death receptors DR4 and DR5 leading to an activation of caspase-8, which cleaves full-length *BID* (BH3 interacting domain death agonist) to its truncated form (*tBID*). tBID is a mediator of mitochondrial damage and in this process cytochrome c is released, which triggers further caspases, such as caspase-9 and caspase-3, inducing apoptosis primarily in tumor cells. In one study (Park et al., 2009) it was shown that a down regulation of PUS10 by RNAi in HeLa cells led to a decrease in TRAIL-induced cell death. Our results showed that the integration of the provirus took place in the third intron of *PUS10.* It is unknown whether this insertion would interfere with the proper transcription and expression of the gene, for example through intron retention (Jacob and Smith, 2017). Intron retention occurs through aberrant splicing and may lead to premature termination codons and thus loss of function. This is especially undesired when tumor suppressors are affected (Jung et al., 2015). Assuming that this mechanism affects the gene *PUS10*, the loss of its function may lead to a growth advantage of the infected cells, since the apoptotic cycle is not properly induced.

SNX25 plays a role in cellular trafficking and signaling and may modulate TGF-β signaling via TGF-β receptors (Hao et al., 2011, Mas et al., 2014). The insertion of the proviral FeLV DNA in exon 7 of this gene may have disrupted its function. However, due to the broad spectrum of functions of this gene, a connection to tumorigenesis is difficult to pinpoint.

TMPRSS5 encodes a protein that is part of the serine protease family. Those proteases are widely spread in the mammalian central nervous system (Mitsui et al., 2008). The expression of *TMPRSS5* was mainly found in the spinal cord and astrocytes of mice (Yamaguchi et al., 2008). To the best of our knowledge it has not been described as an oncogene.

In cat #67 integration sites were all found in-between genes (*GFI1B* and *GTF3C5*, *FAM102A* and *NAIF1, NKX2-2* and *PAX1*) and not within genes.

GFI1B is a transcription factor and required for hematopoiesis. It is a homolog to *GFI1, which* was described as a proviral integration site in T-cell lymphomas in rats caused by the gammaretrovirus Moloney murine leukemia virus (Gilks et al., 1993,

Kim et al., 2003). *GFI1* can also cooperate with the oncogenes c-myc or pim1 and leads to an accelerated onset of lymphomagenesis when overexpressed (Schmidt et al., 1998). But also adverse effects of *GFI1B* are described, so it can act as an important repressor of genes, which function as tumor suppressors (Anguita et al., 2017). Among those genes are also *myc* and *myb* (Rodriguez et al., 2005). *GFI1B* seems also to play a key role in T-lymphopoiesis. Xu and Kee (Xu and Kee, 2007) showed in their study that an ectopic expression of Gfi1b could stop proliferation and survival of T-cell lymphomas in culture obtained from *E2A* (a transcription factor) deficient mice via repression of *GATA3* (transcription factor). So, a loss of its repressor function might lead to malignancies. Due to all those facts it would be interesting to investigate whether *GFI1B* is a preferred integration site in T-cell lymphomas in FeLV-positive cats or otherwise involved in the regulation of T-cell lymphomas in the species cat. ***GTF3C5*** is found next to GFI1B and is involved in RNA polymerase III-mediated transcription (Dumay-Odelot et al., 2007).
NKX2-2 is encoding a transcription factor and is involved in development of the central nervous system and also in glial and neuroendocrine differentiation (Briscoe et al., 1999, Wang et al., 2009). NKX2-2 seems to be a target gene for oncogenic transformation in Ewing's sarcoma (Smith et al., 2006) and has been suggested as an immune histochemical marker to differentiate this tumor from other round cell tumors (Hung et al., 2016).
PAX1 is part of the paired box (PAX) family, which all encodes transcription factors. *PAX1* was found silenced in ovarian and cervical cancer (Su et al., 2009, Huang et al., 2010) and thus is assumed to act as a possible tumor suppressor and is considered a promising biomarker in the diagnosis of malignant and premalignant endometrial lesions (Nikolaidis et al., 2015, Chen et al., 2016).
NAIF1 is involved in apoptosis. It was found down regulated in various human cancers as in non-small cell lung cancer, gastric cancer and prostate cancer (Zhao et al., 2015, Yang et al., 2015, Fu and Cao, 2015). ***FAM102A*** also known as *EEIG1* (early estrogen-induced gene 1) is found next to *NAIF1.* It might be involved in estrogen action and bone homeostasis (Choi et al., 2013).

The two cats #261 and #67 did not share any integration sites; this might be due to distinctive characteristics of the lymphomas. It was reported in a study by Bolin and Levy (Bolin and Levy, 2011) that in other FeLV-infected cats with T-cell lymphomas

predominantly insertion near *c-myc, flvi-2, fit-1 or pim-1* could be observed but not in multicentric, non-T-cell tumors, in which *flvi-1* was identified as CIS (Athas et al., 1995a, Levesque et al., 1990). Although, cat #67 had the typical T-cell lymphoma no integration site near the already described CIS was found, but a new promising site near *GFI1B*, worth investigating. Cat #261 had not the typical FeLV-A T-cell lymphoma but a B-cell lymphoma and therefore the integration sites might be different from common integration sites in FeLV-infected cats. Nevertheless, some sites found in cat #261 might also have relevance in oncogenesis and might be of interest in further studies.

To the best of our knowledge this is the first study to document insertion sites of FeLV-A/Glasgow-1 and its variants. Overall, the most interesting genes in association with lymphoma development in these two cats were in our eyes *GFI1B*, *NAIF1, NKX2-2* and *PAX1* in cat #67 and *NKX2-1* and *DRD2* in cat #261. To assess whether those genes played a key role in tumorigenesis in the tested two cats, further investigation, for example with northern blot would bring more insights into whether these genes are potentially up or down regulated. Southern analysis of genomic DNA from tumor tissue could provide information on the presence of clonally integrated FeLV proviruses, indicative of their involvement during tumorigenesis. Thus, in order to assess the importance of the found insertion sites, southern blot analysis could be performed.

6.2 Replication efficiency

The high abundance of the FeLV progeny variants and a decreased abundance of FeLV-A/Glasgow-1 in cat #261 led to the hypothesis that the variants might have a higher replication efficiency than FeLV-A/Glasgow-1 (Helfer-Hungerbuehler et al., 2010). Our *in vitro* findings do not clearly support this hypothesis. In contrast, the tested variants showed generally lower replication efficiency in FEA cells compared to the original challenge virus FeLV-A/Glasgow-1 with one exception. This observation was more evident when assessing the FeLV p27 antigen levels in the cell culture supernatant and to a lesser degree when quantifying viral RNA. Only one of the tested variants, 261L3, reached replication levels similar to that of FeLV-A/Glasgow-1, not during the early phase after transfection but several days later. The other LTR variants generally showed lower replication capacity in FEA cells compared to FeLV-A/Glasgow-1. In order to confirm that this change in replication efficiency was not due to mutational changes in the LTR acquired during the *in vitro* experiment, the LTRs of FeLV-A/Glasgow-1 and its progenies were re-sequenced at the end of the replication study and the sequence integrity was ensured.

Some LTR variations are associated with a particular disease outcome. Usually, tumor-derived FeLV proviruses collected from natural occurring T-cell lymphomas in cats contain one or more copies of the enhancer (Fulton et al., 1990, Rohn and Overbaugh, 1995, Chandhasin et al., 2004). A particular LTR was found to be common in non-T-cell, non-B-cell lymphoma of the spleen with a single enhancer but with a 21-bp tandem triplication beginning 25 bp downstream of the enhancer (Athas et al., 1995b). In another study a 47-bp tandem triplication in the upstream region of the enhancer was reported that caused myelodysplastic syndrome (MDS) in 41% of the cats experimentally infected with a virus clone carrying this 3 tandem direct repeats; some of these cats eventually developed acute myeloid leukemia (AML) (Hisasue et al., 2009). However, samples for this study describing sequence repeats were taken from naturally infected cats with AML or MDS and it is not clear, whether the triplication occurred de novo in each of the naturally infected cats or alternatively the cats with AML or MDS had all been infected with a virus strain containing the mutation.

All LTRs of the progeny viruses described in this study differed remarkably from the LTR of the original strain FeLV-A/Glasgow-1, but no additional copies of the

enhancer nor other sequence repeats were found and the LTR recombinants did not displayed replication advantage tested on FEA cells. It is difficult to pinpoint the difference in replication efficiency to single mutations in the LTR variants. For example, the variant 261L1 showed mutations in every nuclear protein-binding site, but the replication efficiency was not widely different from those of other variants. Overall, all variants share two specific mutations; one occurred in the enhancer element NF1 and the other one 15 bp upstream of the TATA box, which is part of the core promoter sequence (see Figure 5). Interestingly, the transition from G to A in the location 15 bp upstream of the TATA box was also seen in the sequence of FeLV-C/Sarma, a virus strain that was associated with aplastic anemia in cats (Riedel et al., 1986, Riedel et al., 1988, Tailor et al., 1999, Quigley et al., 2000).

The difference in replication efficiency was particularly pronounced when comparing the *env*-variant with the prototype FeLV-A/Glasgow-1. The envelope protein is particularly crucial for receptor interaction and cell entry. Every FeLV subgroup uses specific host receptors related to differences in the *env* due to recombination and mutations in this gene (Mendoza et al., 2006, Shalev et al., 2009, Takeuchi et al., 1992). Therefore, the mutation in *env* protein preliminary determines disease outcome due to tissue tropisms by way of receptor binding. However, Chandhasin et al. showed that the recombination of 61E/945SL, which contained the *env* and 3' LTR of FeLV-945 in the FeLV-A/61E backbone, not only induced another type of lymphoma but had a small but statistically significant replicative advantage in FEA cells over the 61E/945L recombinant, which only contained the mutated LTR but not env of strain 945 (Chandhasin et al., 2005a). Thus, mutations in *env* predominantly alter disease spectrum but also might provide the virus with a replication advantage. However, the opposite is also possible: mutations in the receptor binding sites may lead to mismatches that interfere with the binding of the receptor and the cell entry may thus be impeded or even impossible. A less efficient cell entry decreases the efficiency of viral replication. Even though the *env* variant was still able to infect feline cell lines in our study, viral RNA loads and p27 antigen levels of the progeny variants were lower compared to FeLV-A/Glasgow-1 in FEA cells. There are several mutations altering amino acid sequences in the *env* of the variants as shown in Figure 4. Important to mention are alterations found in two variable regions (VRA and VRB) within the receptor-binding domain (RBD), in the C2 loop domain and the proline-rich region. VRA and VRB define the specificity for receptor binding (Boomer

et al., 1997, Bolin et al., 2011), thus the mutations and especially the addition of amino acids in VRB might have led to a decreased receptor affinity. The C2 loop is part of the C-terminal region of the FeLV SU, which forms a second RBD in addition to the RBD of the N-terminal region containing VRA and VRB. Even though the C2 loop domain varies naturally between FeLV subgroup *env* genes (Rey et al., 2008b) too many mutations might lead to loss of receptor binding function. The proline-rich region seems not to take part in receptor recognition (Gray and Roth, 1993) but is an important link between the N-terminal region and the C-terminal region (Barnett et al., 2001, Fass et al., 1997) and is said to contribute to viral infectivity (Lavillette et al., 2002). Not only did alteration of the amino acids in the PRR region of the *env* variant occur, but also a loss of three consecutive amino acids. This might also have played a role in the decreased infectivity of the *env* variant of cat #261 in FEA cells.

We cannot exclude the possibility that our variants may have behaved differently if using other cell lines. For example, in a study by Athas et al. the variant 61g945L was tested on FEA cells and on a human malignant hematopoietic progenitor cell line termed K-562 cells, and was observed 4.2 fold more active in K-562 cells than in FEA cells (Athas et al., 1995b, Nishigaki et al., 1997). And in a study by Chandhasin et al. the examined variants were tested on FC6.BM cells, 3201 cells in addition to FEA cells. It was observed that one of the variants showed a statistically significant replicative advantage in FEA cells but did not in the other feline cell lines, when compared to another variant (Chandhasin et al., 2005a). We found some evidence for this in the host range experiment. While FeLV-A/261*env* produced similar levels of p27 antigen in the FEA cells in this study as compared to FeLV-A/Glasgow-1, the *env* variant produced higher p27 antigen levels than the prototype virus in CRFK cells.

Why the progeny variants were more abundant in the tissues of cat #261 is thus not quite clear at present from our *in vitro* results. The vaccination against FeLV and the following regressive course of the FeLV infection after inoculation with the strain FeLV-A/Glasgow-1 might have provided the immune system of cat #261 with an initial protection against FeLV-A/Glasgow-1, which may be in agreement with the persistently high anti-FeLV antibodies that were detected in cat #261 (Helfer-Hungerbuehler et al., 2010). The advanced stage of the FIV infection later on may have been relevant to the course of disease progression; first a progressive FIV infection leads to an immune dysfunction by depleting CD4+ T lymphocytes in

peripheral blood, which possibly facilitated the reoccurrence of FeLV in cat #261 (Pedersen et al., 1989, Magden et al., 2011, Ravi et al., 2010, Hoffmann-Fezer et al., 1992, Hofmann-Lehmann et al., 1997). Second, it is suggested that during the course of an FIV infection a loss in immune surveillance function occurs (Callanan et al., 1992, Callanan et al., 1996, Hartmann, 2011, Terry et al., 1995, Magden et al., 2011). This might have given an “escape” advantage to the variants compared to the original inoculation strain FeLV-A/Glasgow-1, which was already “known” to the immune system.

6.3 Importance of co-infections with other cancer associated viruses

6.3.1 FIV co-infection in cat #261

Interestingly, the cat #261 developed a multicentric monoclonal B-cell lymphoma (Helfer-Hungerbuehler et al., 2010). The co-infection of FIV in cat #261 might also have played an important role in the development of this tumor phenotype (large B-cell lymphoma), which was associated with FIV infection (Callanan et al., 1996, Terry et al., 1995, Wang et al., 2001, Gabor et al., 2001). Early on an association between FIV and an increased incidence of neoplasia was assumed (Callanan et al., 1992, Gabor et al., 2001, Magden et al., 2011, Pedersen et al., 1989, Terry et al., 1995, Poli et al., 1994, Shelton et al., 1990). But only in few cases a direct oncogenic role of FIV could be shown in the emergence of neoplasia (Beatty et al., 1998, Beatty et al., 2002, Wang et al., 2001). It is thought that the mechanism of tumorigenesis occurs in a more indirect way (Callanan et al., 1992, Callanan et al., 1996, Hartmann, 2011, Terry et al., 1995, Magden et al., 2011) by an impaired immune surveillance and failure to eliminate neoplastic cells due to vast expansion of lymphoid cells. Studies by Callanan et al. (Callanan et al., 1992, Callanan et al., 1996) showed an increased B cell proliferation in FIV infected cats (Flynn et al., 1994). They suggested that would lead to more opportunities of malignant transformation. The indirect mechanisms of FIV providing a greater pool of B cells in combination with an immunosuppression might have facilitated the transformation of a single B-cell - with or without the assistance of FeLV - thereby leading to a diffuse monoclonal B-cell lymphoma in cat #261 (Helfer-Hungerbuehler et al., 2010).

6.3.2 Other cancer associated viral co-infections in humans and cats

Co-infections with other viruses are also associated with a higher likelihood of cancer development. For example, the two human gammaherpesviruses (GHV), Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated virus (KSHV), were associated with specific kinds of lymphomas in HIV positive humans (Thompson et al., 2004, Gloghini et al., 2013). Recently a feline gammaherpesvirus was discovered, which is also under investigation for a possible tumorigenic potential especially in FIV infected cats (Troyer et al., 2014, Beatty et al., 2014, Tateno et al., 2017, McLuckie et al., 2017, Kaye et al., 2016, McLuckie et al., 2018). Given the discovery of this new virus,

both cats used in this study (#261 and #67) were retrospectively tested for feline gammaherpesvirus by real-time PCR (Troyer et al., 2014): both cats tested negative for the feline gammaherpesvirus at the time of necropsy (data not shown).

6.4 Host Range

Different subgroups of FeLV bind to specific receptors, which may be ecotropic, as is the case with the FeLV-A receptor THTR1, or amphotropic, as is the case with the FeLV-B receptor Pit1 and Pit2 (Anderson et al., 2001). The host range experiment of this study was performed in order to review the *env*-variant to provide more information whether the mutations of this variant expand the ecotropic host range of FeLV-A/Glasgow-1.

The replication efficiency of the *env*-variant in FEA cells was lower compared to that of FeLV-A/Glasgow-1. Thus, a titration experiment was conducted prior to the main host range experiment to yield virus preparations with similar infectivity. For the FeLV-A/261*env* variant a virus stock could be produced in FEA cells; the maximal achievable virus concentration was lower than that of FeLV-A/Glasgow-1. In consequence, the stock solution of FeLV-A/Glasgow-1 had to be diluted tenfold to approximately match the infectious dose of the *env*-variant. Moreover, FeLV-A/Glasgwo-1 was also tested undiluted, as the low MOIs might have led to a loss of infectivity. Using a virus concentration kit might have been an option to concentrate the virus. We refrained from using this option for the present study because of previous poor results caused by massive loss of viral infectivity during the procedure.

In accordance to previous reports FeLV-A/Glasgow-1 could not infect HeLa, ST-IOWA, 104C1 and *M. dunni* cell lines (Nakata et al., 2003, Moser et al., 1998, Shalev et al., 2009). This also applied to the variant FeLV-A/261*env*, which was unable to infect these cell lines.

Nakata et al. showed that an infection of HEK293 cells with FeLV-A/Glasgow-1 was possible but with a very low titer of expression (4,050 focus forming units/ml of lacZ pseudotype) (Nakata et al., 2003). This observation applies to our findings, where FeLV-A/Glasgow-1 could successfully infect HEK293 cells using the stock solution, while the tenfold dilution and the stock solution of FeLV-A/*261env* failed to induce a productive infection due to low MOIs.

The feline cell lines (FEA and CRFK), were, as expected, infected by both virus strains. This indicates no difference in the host cell spectrum using feline cells. Remarkably, the infection of FEA cells showed no differences in p27 antigen levels when using virus infection and comparable MOIs of the different viruses, while the *env* variant produced higher p27 antigen levels in CRFK cells than the FeLV-

A/Glasgow-1 virus. This indicates a difference between the original challenge strain FeLV-A/Glasgow-1 and its progeny FeLV-A/261*env* in replication efficiency in CRFK cells.

MDCK cells were shown to be susceptible to FeLV-B but not to FeLV-A (Nakata et al., 2003, Shojima et al., 2006). In an older study a long-term infection of MDCK cells with FeLV could be induced but the paper did not state whether the virus used in the experiment is of the subgroup A or B (Essex et al., 1972). Unexpectedly, in our study we observed an infection of MDCK cells with FeLV-A/Glasgow-1. An infection was also observed with the lower virus titer of FeLV-A/Glasgow-1 but not with the recombinant FeLV-A/261*env* variant. In order to confirm the exclusive presence of dog cells in the MDCK cells and absence of any cat cell contamination or cell-line confusion, a feline albumin gene PCR was performed (Kessler et al., 2009), which recognizes cells of feline origin. No feline albumin could be detected in the MDCK cells. The possibility that the MDCK cell line used in this study differs from the one used in other studies is not entirely excluded, since the origin of the cell line could not be traced anymore.

Finally, the result of the infection of the MDCK and also of the CRFK cell lines might indicate a difference between the virus strain FeLV-A/Glasgow-1 and the recombinant *env*-variant. A closer look to receptor usage might bring more light into this matter. An interference assay (Sarma and Log, 1973) could be carried out to verify whether the recombinant *env* variant truly belongs to the FeLV subgroup A. Even though some further experiment could provide more in-depth information about the recombinant variant FeLV-A/261*env*, we conclude that the mutational changes in this variant did not expand the ecotropic host range of the FeLV subgroup A.

6.5 Conclusions

To the best of our knowledge, this is the first time that insertion sites of FeLV-A/Glasgow-1 and progeny viruses thereof are described. The integration sites found for FeLV-A/Glasgow-1 in cat #261 and its variants, as well as the integration sites in cat #67 were not described in cats so far. Several of the identified genes, where the insertions were located (particularly *GFI1B*, *NAIF1, NKX2-2, PAX1*, *NKX2-1*), are of interest due to their role in the apoptosis cycle and relation to tumorigenesis recognized in other species. A homolog of one of these genes (*GFI1*) had been recognized earlier as an integration site of the gammaretrovirus Moloney murine leukemia virus in a rat with a T-cell lymphoma. The integration sites in the two investigated cats, one with a B-cell lymphoma and a FIV coinfection (cat #261) and one only FeLV-A/Glasgow-1 infected and with a T-cell lymphoma, were distinct. Further studies will be necessary to investigate the importance of these insertion sites in the pathogenesis of FeLV-associated lymphoma.

In the immunodeficient cat #261 highly divergent and *in vivo* dominant FeLV-variants developed during the course of FeLV-infection. Some of these variants were further investigated for *in vitro* replication capacity and host range. The host range experiment of the envelope variant showed the ecotropic limit, which is typical for FeLV-A subgroups, so that the mutational changes in the envelope did not expand the host range, like it would be seen in FeLV-B subgroups. Despite the higher loads of the FeLV variants in most of the tissues investigated of cat #261, the replication efficacies of the variants were largely lower in FEA cells compared to the original inoculation strain FeLV-A/Glasgow-1. These findings though cannot exclude possible cell-type specific behaviors of the variants in other cell lines. However, the abundance of the progeny variants in the tissue of cat #261 in comparison of the original inoculation strain FeLV-A/Glasgow-1 might be closer attributed to the co-infection with FIV than a mutational increased viral fitness of the variants. Thus, it is our hypothesis that the variants that developed during the course of the infection in the highly immunosuppressed, CD4+ depleted cat #261 became predominant due to their potential evasion to the immune system.

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8 Annex

Feline leukemia virus strain Glasgow-1, complete genome

GeneBank: KP728112.1

TGAAAGACCCCCTACCCCAAAATTTAGCCAGCTACTGCAGTGGTGTCATTTCACAAGGCATGGAAAATTA
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